Genetic and physiological aspects of barley (*Hordeum vulgare* L.) responses to waterlogging, salinity and aluminium stresses and their interactions

by

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Declarations

The thesis contains no material which has been accepted for the a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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This thesis was completed during the course of my enrolment in a PhD degree in the School of Land and Food at the University of Tasmania. This thesis contains no experimental results that have previously presented for any degree at this or other institution.

This thesis contains one literature review chapter and three main research chapters. One section in the literature review chapter (Chapter 2) has been published as a book chapter. Results described in the three research chapters have been published in different journals.

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Fan Y, Shabala S, **Ma YL**, Xu R, Zhou MX. Using QTL mapping to investigate the relationships between abiotic stress tolerance (drought and salinity) and agronomic and physiological traits. BMC Genomics, 2015, **16:** Article 43

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Manuscripts in preparation:

Ma YL, Habib A, Liu CJ, Shabala L, Zhou MX, Shabala S. The role of ROS and calcium in crown rot resistance under drought conditions in barley.

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Ma YL, Li CD, Johnson PG, Zhou MX. Combining ability of acid soil tolerance in barley. The 16th Australian Barley Technical Symposium, 8-11 September 2013, Melbourne, Australia

Abstract

The term “waterlogging” is used to describe stressful conditions of root environment with excessive water, under which the diffusion of gases is reduced by almost four times of magnitude compared with that in the air. Decreased water and nutrient absorption by roots occurs with either complete (anoxia) or partial (hypoxia) depletion of oxygen. Waterlogging events may be a result of prolonged rain, flooding caused by melting snow, or poor soil drainage. The yield loss caused by waterlogging varies with duration of the stress, soil types and the tolerance of different species.

The waterlogging stress could occur simultaneously with other soil stresses such as salinity which is another major abiotic stress that limits crop production via adverse effects of osmotic stress, specific ion toxicity, and stress-related nutritional disorders. Detrimental effects of salinity are often exacerbated by low oxygen availability when plants are grown under waterlogged conditions. In our study, we investigated the interaction between waterlogging and salinity stresses. A doubled haploid (DH) population consisting of 175 lines derived from a cross between a Chinese barley (*Hordeum vulgare* L.) variety Yangsimai 1 (YSM1) and an Australian malting barley variety Gairdner was used to construct a high density molecular map which contained more than 8,000 Diversity Arrays Technology (DArT) markers and single nucleotide polymorphism (SNP) markers. Salinity tolerance of parental and DH lines was evaluated under drained (SalinityD) and waterlogged (SalinityW) conditions at two different sowing times. Three quantitative trait loci (QTL) located on chromosome 1H, single QTL located on chromosome 1H, 2H, 4H, 5H and 7H, were identified to be responsible for salinity tolerance under different environments. Waterlogging stress, day length and temperature showed significant effects on barley salinity tolerance. The QTL for salinity tolerance mapped on chromosomes 4H (*QSlwd.YG.4H*) and 7H (*QSlwd.YG.7H* and *QSlww.YG.7H*) were only identified in winter trials, while the QTL on chromosome 2H (*QSlsd.YG.2H* and *QSlsw.YG.2H*) were only detected in summer trials. Genes associated with flowering time were found to pose significant effects on the salinity QTL mapped on chromosomes 2H and 5H in summer trials. Given the fact that two QTL for salinity tolerance on 1H, *QSlsd.YG.1H* and *QSlww.YG.1H-1*, reported

here have never been considered in the literature, this warrants further investigation and evaluation for suitability to be used in breeding programs.

Aluminium (Al) is prevalent in soils, but Al toxicity is manifested only in acid conditions. It causes severe damages to the root system. Short-term waterlogging stress can occur simultaneously with Al toxicity in areas with high rainfall or inappropriate irrigation pattern. In this work, we investigated effects of short-term treatments with hypoxia and phenolic acid (two major constraints in waterlogged soils) on roots' sensitivity to low-pH and Al stresses. We showed that hypoxia-primed roots maintained higher cell viability when exposed to low-pH/Al stress, in both elongation and mature root zones, and superior ability to retain K^+ in response to low-pH/Al stresses. These priming effects were not related to higher H^+-ATPase activity and better membrane potential maintenance, and could not be explained by the increased expression levels of *HvHAK1*, which mediates high-affinity K^+ uptake in roots. Instead, hypoxia-conditioned roots were significantly less sensitive to H_2O_2 treatment, indicated by a 10-fold reduction in the magnitude of K^+ efflux changes. This suggested that roots pre-treated with hypoxia desensitised ROS (reactive oxygen species)-inducible K^+ efflux channels in root epidermis and had enhanced anti-oxidative capacity. A possible role of Ca^{2+} in stress-induced ROS signalling pathways is also discussed. We report, for the first time, the phenomenon of cross-protection between hypoxia and low-pH/Al stresses, and causally link it to cell's ability to maintain cytosolic K^+ homeostasis.

Barley is one of the most Al-sensitive small-grained cereals. The major gene for Al tolerance in barley is *HvAACT1* (*HvMATE*) on chromosome 4H which encodes a multidrug and toxic compound extrusion (MATE) protein. The HvAACT1 protein facilitates the Al-activated release of citrate from root apices which protects the growing cells and enables root elongation to continue. A 1-kb transposable element-like insertion in the 5'-untranslated region (UTR) of *HvAACT1* is associated with increased gene expression and Al tolerance and a PCR-based marker is available to score for this insertion. We screened a wide range of barley genotypes for Al tolerance and identified a moderately tolerant Chinese genotype named CXHKSL which did not show the typical allele in the 5'-UTR of *HvAACT1* that is associated with tolerance. We investigated the mechanism of Al tolerance in CXHKSL and concluded that it also relies on the Al-activated release of citrate from roots. Quantitative trait loci (QTL) analysis

of doubled haploid lines generated with CXHKSL and the Al-sensitive variety Gairdner mapped the tolerance locus to the same region as *HvAACT1* on chromosome 4H. We found that the Chinese barley genotype CXHKSL possesses a novel allele of the major Al tolerance gene *HvAACT1*.

In conclusion, a novel allele of the major Al tolerance gene *HvAACT1* was identified from a Chinese variety. The allele also relies on the Al-activated release of citrate from roots. In addition, significant interactions between various stresses exist. Waterlogging stress increased the severity of salinity stress, while short-term waterlogging stress elevated barley tolerance to Al toxicity through ROS and Ca^{2+} signallings. Environmental conditions, for example, day length and temperature could pose significant effects on salinity tolerance. Therefore, both of those external (growth temperature, day length and soil water level) and internal factors (plant flowering time, cytosol ROS production, cytosol Ca^{2+} levels and allelic variation of tolerance genes) should be given significant emphasis when evaluating barley tolerance to either a specific abiotic stress or combined stresses.

List of Abbreviations

ABA	abscisic acid
AHA	*Arabidopsis* H^+-ATPase
Al	aluminium
AlC	aluminium -conditioned
ALMT	aluminium-activated malate transporter
BSM	basic sodium medium
CAPS	cleaved amplified polymorphic sequence
DACC	depolarization-activated Ca^{2+} channels
DHL	double haploid lines
EZ	elongation zone
FDA	fluorescein diacetate
FT	flowering time
GORK	guard cell-type outward-rectifying K^+ channels
HAC	hydroxybenzoic-acid conditioned
HACC	hyperpolarization-activated Ca^{2+} channels
HAK	high-affinity K^+ transporter
HC	hypoxia conditioned
IM	interval mapping
INDEL	insertion-deletion
LOD	logarithm of the odds
LOES	low-oxygen escape syndrome
LOQS	low-oxygen quiescence syndrome
MATE	multidrug and toxic compound extrusion
MIFE	non-invasive microelectrode ion flux measurements
MP	membrane potential
MQM	multiple QTL model
MZ	mature zone
NC	non-conditioned
NIL	near-isogenic line
NSCC	non-selective ion channels
nsHbs	non-symbiotic hemoglobins
PCD	programmed cell death
PCR	polymerase chain reaction
PI	propidium iodide
PM	plasma membrane
PMHA	plasma membrane H^+-ATPase
SD	salinity with drained conditions
SNP	single nucleotide polymorphism
SW	salinity with waterlogged conditions
QTL	quantitative trait loci
RILs	recombinant inbred lines
RT-PCR	quantification real time polymerase chain reaction
ROL	radial oxygen loss
ROS	reactive oxygen species
TFs	transcription factors
UTR	untranslated region
YSM1	Yangsimai 1

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Chapter 1 Introduction

1.1 Background information

1.1.1 Waterlogging stress

Waterlogging, as a world-wide stress, adversely affects about 10% of the global land area (Setter and Waters, 2003) and reduces crop production by as much as 80% (Shabala, 2011). Under waterlogging stresses, the soil gas exchange is severely repressed. This leads to a significant depletion of free oxygen (Bailey-Serres and Voesenek, 2008). As soon as the free O_2 surrounding the roots is depleted or restricted, hypoxia stress occurs. Hypoxia can cause a transfer from aerobic to anaerobic metabolism in roots and significantly suppress ATP synthesis (Barrett-Lennard, 2003). Waterlogging also results in a sharp decrease in the soil redox potential, resulting in very significant changes to the soil chemical profile. Those effects include a changed availability of mineral substances, increased solubility of potentially toxic metals and accumulation of toxic compounds (Shabala, 2011). For example, the phenolic acids, as toxic secondary metabolites, could become injurious in the low-pH rhizosphere and further reduce the pH values of waterlogged soil (Greenway et al., 2006, Pang et al., 2007, Shabala, 2011). Meanwhile, excessive reactive oxygen species (ROS) generation in plant cells is an integral part of many abiotic stresses (Mittler, 2002). Elevated ROS production is also unavoidable for plants under waterlogging stress (Li et al., 2012). Increased hydrogen peroxide or superoxide anion radical accumulation has been observed in hypoxia-stressed pigeon pea (*Cajanus cajan* L.) leaves (Sairam et al., 2009) and wheat roots (Biemelt et al., 2000).

1.1.2 Waterlogging stress and salinity stress

Although waterlogging is a widespread stress in its own right, it can also be an additional soil constraint in landscapes affected by salinity (Barrett-Lennard, 2003, Bennett et al., 2009). In such landscapes waterlogging may occur because the salinity either coincides with a shallow water table or there is decreased infiltration of surface water (Jenkins et al., 2010). The occurrence of combined salinity and waterlogging stress can result from intensive irrigation in agricultural production systems, rise of

saline water tables, and seawater intrusion in coastal environments (Zeng et al., 2013). In recent years, an impressive amount of knowledge has accumulated on plant physiological and molecular responses to salinity or waterlogging stresses. However, studies dealing with the combined effects of these two stresses are much rarer and often controversial (Setter and Waters, 2003). Those studies on the interaction between waterlogging and salinity stress are focusing on the physiological mechanisms for non-halophytes, halophytes and salt-land pasture production (Barrett-Lennard, 2003, Barrett-Lennard and Shabala, 2013, Bennett et al., 2009, Colmer and Flowers, 2008). The characteristic response for most dryland plants is that root zone hypoxia exacerbates the effects of salinity by increasing the concentrations of toxic ions (Na^+ and Cl^-) rapidly in shoot tissues, which impacts on plant growth and survival (Barrett-Lennard and Shabala, 2013, Jenkins et al., 2010).

1.1.3 Waterlogging stress and aluminium toxicity

Aluminium (Al) toxicity is the major factor limiting crop production in acid soils. Al hydrolyses in solution such that the trivalent Al species, Al^{3+}, dominates in acid conditions (pH < 5) (Delhaize and Ryan, 1995). Around 35% of the world's arable land is acid soil and nearly 50% is potentially arable land (Ryan et al., 2011). Soil acidity with high levels of toxic Al is the largest (in area) soil constraint limiting sustainable crop production in Australia. At least 50 million hectares of surface soil, and 23 million hectares of subsoils, are acidic in Australia. In Western Australia (WA), about two third of wheat-belt soils are either acid or at risk of soil acidification. Currently soil acidity is estimated to cost WA grain growers between $300–400 million annually. At low concentrations, Al inhibits root elongation, resulting in root structural and functional damage (Kochian, 1995). The subsequent restriction of root extension and surface area limits root growth in the soil horizon, predisposing plants to reduced productivity (Abdel-Haleem et al., 2014, Kochian et al., 2004). Soil acidification is closely associated with soil waterlogging in high rainfall regions, which is one of the major restrictions for barley production. There are large areas of waterlogging-prone agricultural land in Australia, including 1.8 million ha in WA (with > 400mm annual rainfall) and 4.1 million ha (mostly duplex soils) in Victoria in Australia, with an estimated yield loss of 50%-85% in barley. In perspective of the ion toxicity, it was found that waterlogging could increase concentrations of Al in acidic soils, which was

linked with an increase in shoot concentrations of Al in wheat genotypes (Khabaz-Saberi and Rengel, 2010). Tolerance to Al toxicity has been shown to improve grain yield in transiently waterlogged soils in some wheat genotypes (Khabaz-Saberi et al., 2014). It has been assumed that the priming effects of hypoxia stress are mainly driven by (i) an enhanced activity of key enzymes in alcohol fermentation (ADC and PDC); (ii) the capability to maintain a higher level of respiration, evidenced by a lesser decrease in heat development and ATP production; and (iii) the maintenance of a better ion homeostasis and K^+ channel function (Mugnai et al., 2011). However, no studies for the plausible mechanism underlying the effects of short-term waterlogging (hypoxia) on Al tolerance have been conducted yet.

Lime is typically used to neutralize soil acidity, by elevating the pH and buffering it against re-acidification processes. However, as lime typically takes decades to move downward into the acidic subsoil, those applications only correct Al toxicity problems in the topsoil (Caires et al., 2008). An environmentally friendly alternative is to develop tolerant cultivars which have deeper rooting and greater root function in the presence of toxic subsoil Al (Abdel-Haleem et al., 2014, Hiradate et al., 2007). In general, plants can cope with Al toxicity via two mechanisms: (1) exclusion of Al from root cells, particularly cells in the meristematic regions (Silva et al., 2010), by producing and excreting chelating chemicals (Kochian et al., 2004); or (2) accumulation of Al and internal detoxification, again probably involving chemical chelation (Abdel-Haleem et al., 2014, Delhaize et al., 2012, Watanabe et al., 2005). The physiology and genetics of Al tolerance mechanisms involving organic anion (malate or citrate acid) efflux from roots have now been investigated in a range of species, for example, wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Delhaize et al., 2012). Over the past decade, genes encoding these and other newly discovered mechanisms of tolerance have been cloned. The key features of Al tolerance gene expression constitutively higher gene expression occurs in wheat and barley for the Al resistance genes *TaALMT1*, *TaMATE1B*, and *HvAACT1* (Kochian et al., 2015). For the plant species where the *ALMT* and *MATE* genes are constitutively expressed, malate and citrate efflux occurs with no discernible lag between root Al exposure and organic acid release (Kochian et al., 2015).

1.2 Statement of gaps in previous research

It is suggested that a salt-tolerant species would also have a higher tolerance to the combined waterlogging and salinity stress than a salt-sensitive one (Turkan et al., 2013). Previous studies mostly focused on the physiological mechanisms in Na^+/K^+ homeostasis, ATPase activity and membrane H^+ gradients (Barrett-Lennard and Shabala, 2013, Zeng et al., 2013). Keeping in mind that both the salinity tolerance and waterlogging tolerance are complex quantitative trait controlled by multiple major or minor genes (Mano and Takeda, 1997, Qiu et al., 2011, Wu et al., 2012, Xue et al., 2009), the current research intended to identify QTL conferring the salinity tolerance of barley under waterlogged conditions using a newly-developed double haploid population. On the other hand, the control of flowering time in cereals (also referred as heading time) and grasses has been intensively studied and key genes have been identified in the vernalisation (low temperature) and photoperiod (daylight length) response pathways (Asp et al., 2011, Trevaskis et al., 2007). Barley is classified as a long-day cereal, and its flowering time could be promoted when exposed to long photoperiod conditions (Faure et al., 2007). Meanwhile, as a temperate cereal, the flowering time of barley is accelerated by prolonged exposure to non-freezing cold (vernalisation), especially for the winter-type genotypes (Casao et al., 2011, Hemming et al., 2008). In perspective of the interaction between flowering time and stress tolerance, it was demonstrated that in winter wheat and barley varieties, long exposures to non-freezing cold temperatures accelerate flowering time (vernalisation) and improve freezing tolerance (cold acclimation) (Dhillon et al., 2010). However, few previous studies have reported the interaction between flowering time and salinity / waterlogging tolerance in barley (Diallo et al., 2014). The timing of floral transition is associated with the heading time in cereal crops and constitutes an important character for adaptability to various environmental conditions (Yaish et al., 2011). This study aimed to explore the potential effects of flowering time (which is associated with growth temperature and daylight length) on plant tolerance to salinity and waterlogging stresses.

Although *HvAACT1* gene has been identified as the major aluminium tolerance gene in barley, which encodes a MATE transporter, diversity of Al tolerance exists within species (Bian et al., 2013). Meanwhile, the Al-tolerance is also associated with the

polymorphisms of MATE genes. Transposable elements located at the 5'-UTR are capable of altering *HvAACT1* gene expression (Delhaize et al., 2012). Therefore identifying and developing Al-tolerant germplasms with diverse aluminium tolerant genes/alleles are more plausible. On the other hand, although aluminium toxicity with soil acidity that is associated with soil waterlogging is one of the most severe conditions in the soil environments, no studies for the mechanism underlying the effects of short-term waterlogging (hypoxia) on Al tolerance have been conducted previously. In field conditions, investigating the interaction between waterlogging stress and Al toxicity is difficult due to dynamic properties of soil structure and soil components, which can significantly affect the severity and duration of Al toxicity. In this study, to overcome those problems we used hydroponics culture methods to simulate field conditions and hypoxia treatments to represent for field waterlogging stress. A controllable environment was set up with those methods, and facilitated the study of physiological mechanisms in stress tolerance. Moreover, recent studies have shown the significant role of reactive oxygen species (ROS) in stress cross-talk (Pottosin et al., 2014, Steffens, 2014). Under abiotic stress conditions, ROS levels are commonly elevated compared to pre-stress levels (Mittler, 2002). ROS can play a key role as signal transduction molecules involved in mediating responses to pathogen infection, environmental stresses, programmed cell death and different developmental stimuli (Mittler et al., 2004, Torres and Dangl, 2005). Accumulation of H_2O_2 in the apoplast is thought to be involved in acclimation responses of plants to drought and salt stresses, such as growth and cell wall strengthening (Miller et al., 2010). Those assumptions/ results on roles of ROS provide avenues for investigating the possible mechanism for the interaction between waterlogging tolerance and Al tolerance.

1.3 Aims of the project

The objectives of this research are to study the plant tolerance performance under different stress conditions and investigate the mechanisms underlying tolerance to combined stresses.

1. To identify the genetic tolerance mechanism for salinity stress when waterlogging conditions are applied and evaluate the effects of other environmental factors (temperature/ daylight length) on plant stress tolerance.

2. To identify the role of ROS in the interaction between waterlogging and Al tolerances
3. To identify new allele of the major Al tolerance gene *HvAACT1* in barley.

1.4 Brief description of the methodology

For the phenotyping work, leaf chlorosis and plant survival rate were scored to evaluate the salinity and waterlogging tolerance in the experiments conducted under glasshouse and field conditions with 2~3 independent trials. In addition, the contents of leaf Na and K and the ratio of Na/K were also measured for salinity tolerance. For assessing the Al tolerance, we used root length, damage of root tip, and citrate/malate flux as tolerance indices. The Al tolerance was evaluated under both soil and hydroponics conditions.

Molecular techniques used in this study include molecular marker development, standard PCR and real-time quantitative PCR. To identify quantitative trait loci conferring the stress tolerance, softwares JoinMap 4.0 and MapQTL6 were used for analysing the obtained phenotypic and molecular data from segregating mapping populations. In the physiological experiments the techniques of cell viability staining, ion flux measurement and membrane potential measurement was applied in studying the interaction between waterlogging tolerance and Al tolerance. To simulate the field waterlogging stress, we used 0.2% agar-added and N_2-bubbled solution to establish a low-oxygen (hypoxia) stress, which is the main adverse effect of short-term waterlogging stress.

1.5 Outlines of chapters (thesis plan)

Chapter 2---Literature review
Chapter 3---Quantitative trait loci for salinity tolerance under waterlogged and drained conditions and their association with flowering time
Chapter 4---Roots conditioning with hypoxia increase aluminium and acid stress tolerance by mitigating activation of K^+ efflux channels through ROS in barley: insights into cross-tolerance mechanisms
Chapter 5---Allelic variation of the major Al tolerance gene *HvAACT1* in barley
Chapter 6---General conclusions and future prospects

Chapter 2 Literature review

2.1 Waterlogging stress

The term ‘waterlogging’ is used to describe stressful conditions of root environment with excessive water, under which the diffusion of gases is reduced by almost four times of magnitude compared with that in the air. Decreased water and nutrient absorption by roots occurs with either complete (anoxia) or partial (hypoxia) depletion of oxygen (Malik et al., 2002). Waterlogging events may be a result of either a prolonged rain, flooding caused by melting snow, or poor soil drainage. The yield loss caused by waterlogging is varying with duration of the stress, soil types and the tolerance of different species (Bailey-Serres and Voesenek, 2008). In the USA alone 16% of soils are influenced by waterlogging, and economic penalties for crops are estimated to be the second largest after drought stress (Zhou, 2011). The definition of the waterlogging tolerance is either plant survival or maintenance of relative growth rate, grain yield or biomass accumulation under waterlogging compared with non-waterlogged conditions, or both. Waterlogging can cause damage to dryland plants even when water level is lower than soil surface (Malik et al., 2001) or when transient (e.g. days) (Malik et al., 2002). Submergence is used to describe the situation when most or all aerial tissues or organs are under water. Submergence blocks direct exchange of gases between the entire plant body and air resulting in decreased O_2 and CO_2 levels (Colmer and Voesenek, 2009).[1]

2.1.1 Adverse effects of waterlogging stress

Free water is essential for the growth of all higher plants. However, excess water in the root zone of land plants is detrimental or lethal when it forms a barrier between soil and air free transfer of gases, such as O_2 and CO_2 (Drew, 1997), with the effect of inadequate oxygen supply being most significant. Symptoms of waterlogging injury (especially for young plants) similar to waterlogged soil may be also developed in hydroponic culture

[1] The content of Section 2.1 has been accepted as a book chapter for publication: **Ma YL,** Zhou MX, Shabala S, Li CD. Exploration and utilization of waterlogging-tolerant barley germplasm. In: Zhang GP, Li CD eds. Exploration, Identification and Utilization of Barley Germplasm. 2015. Elsevier

lacking proper aeration (Trought and Drew, 1980). Fully submerged plants may also experience shading stress (Sarkar and Panda, 2009).

2.1.1.1 Suppression of plant growth

Seed germination and survival rates of crops could be severely reduced under low oxygen condition due to limited ability of using the stored starch reserves and soluble sugars (Ismail et al., 2009). Low activity of amylase, high activity of peroxidase and insufficient ethylene are responsible for that ineffective starch catabolism. Moreover, levels of sucrose synthase transcript increase while levels of sucrose invertase decrease, indicating that sucrose synthase is probably the principal enzyme that converts sucrose to phosphorylated hexoses under low-oxygen stress (Geigenberger, 2003). Post-effects of short-term waterlogging were evident for wheat shoots at recovery stage, including accelerated leaf senescence, reduced final leaf elongation, smaller final leaf area, fewer tiller number and decreased biomass (Malik et al., 2002). O_2 deficiency during waterlogging leads to the reduced availability of energy in the roots (Thomson et al., 1990) and, as a result, energy-dependent processes such as nutrient uptake are inhibited (Setter and Belford, 1990). N deficiency was believed to be the principal cause for the suppression of growth since tiller number was reduced by 50% at the end of the waterlogging stress which resulted in a significant yield loss. Grain yield was regained by 20% when N was applied after waterlogging stress. However, this promotion effect on yield was not invoked when high N was applied at the sowing stage (Robertson et al., 2009). Root biomass of barley seedlings was reduced during waterlogging treatment (Pang et al., 2004) and recovered when short-term hypoxic stress was relieved. That recovery could be explained by initiation of new nodal roots and proliferation of lateral shoots on these nodal roots (Malik et al., 2002). The respiration rate and O_2 uptake are also significantly reduced along the whole root during the hypoxia. However, functionally active tissues in root apex still display net O_2 influx in contrast with mature and elongation zones (Pang et al., 2006). When exposure to waterlogging stress occurred at late developmental stage (heading or just before flowering), the root respiration rate recovery was slower than that of seedling stage (Araki et al., 2012). In water-submerged conditions, plants are also more prone to be affected at early developmental stages, and leaf elongation in younger leaves was faster than older leaves. This phenomenon was explained by the high-light and low nutrient absorption which

contributed to the non-structural carbohydrates accumulation. Higher carbohydrates reserves in younger leaves consequently induced faster leaf elongation under submergence (Huber et al., 2012).

2.1.1.2 Energy shortage

Waterlogged organs must increase catabolic rate to produce more ATP when anaerobic ATP production is repressed in anoxic cells (Bailey-Serres et al., 2012). Some ATP can be synthesized in glycolysis when NAD^+ is regenerated in the form of conversion of pyruvate to ethanol. The amount of ATP produced by glycolysis is much less than by oxidative phosphorylation, which leads to the occurrence of 'energy crisis' (Gibbs and Greenway, 2003, Kulichikhin et al., 2009). Carbohydrate (the energy reserve) production is reduced dramatically during complete submergence due to reduced photosynthesis rate during the periods of submergence or subsequent de-submergence. There are three major factors that affect the photosynthesis rate: 1) Levels of chlorophyll contents dropped during submergence (Sarkar et al., 2001). 2) The decrease of Rubisco activity resulted in poor CO_2 photosynthetic rate. Rubisco activity was not able to recover to a normal level even when photosynthetic apparatus recovered after de-submergence. 3) Reduced energy supply from supressed aerobic respiration also posed a detrimental effect on susceptible rice plants (Sarkar et al., 2006). Several wetland plants displayed different responses in shoots during submergence and in the recovery of photosynthetic activity after re-emergence. These include *Phragmites australis* (Mauchamp et al., 2001) and *E.cellulosa* (Macek et al., 2006), and it was suggested that these species might facilitate gas diffusion and make use of under-water photosynthesis to avoid the damage (Mommer et al., 2006).

2.1.1.3 Increased ion and metabolite toxicities

The decrease in redox potential induced by flooding increases the accumulation and solubility of many toxic metals, including manganese, iron, sulphur and phosphorus (Jackson and Drew, 1984). Apart from the elemental toxicities to the sensitive root tips, increased concentration of secondary metabolites such as phenolics and volatiles fatty acid could become injurious in the low-pH rhizosphere (Pang et al., 2007, Shabala, 2011). pH values of waterlogged soil can be further reduced by the accumulation of

volatile organic acids as well as the high concentration of CO_2 (Greenway et al., 2006). Another potential toxic metabolite found in waterlogged soil is ethylene, which supresses root growth. In addition, with the re-introduction of oxygen at the recovery phase, the remaining ethanol in anoxic cells will be transformed into acetaldehyde which may injure cells (Bailey-Serres and Voesenek, 2008). Waterlogging also often occurs in saline soils, and it was demonstrated that over-accumulation of Na^+ and Cl^- in shoots under waterlogged conditions is greater than for salinity alone since energy (ATP) crisis caused by waterlogging-induced anoxic stress dampened the exclusion of Na^+ and Cl^- (Teakle et al., 2010). Higher concentration of Na^+ and Cl^- also reduced water uptake and resulted in a severe reduction in the uptake of K^+ (Malik et al., 2009).

2.1.1.4 Elevated reactive oxygen species (ROS) production

ROS in plants are formed as by-products of aerobic metabolism (Li et al., 2012). Under many abiotic stresses, ROS levels are always elevated compared to pre-stress levels (Mittler, 2002). Excessive production of various ROS such as superoxide radicals, hydroxyl radicals, hydrogen peroxide and singlet oxygen, were found in hypoxia-stressed leaf and root tissues (Blokhina et al., 2003, Sairam et al., 2009). To avoid ROS-induced damage, efficient ROS scavenging systems are established in plant cells. These include both enzymatic and non-enzymatic antioxidant systems. Enzymatic antioxidant system includes superoxide dismutase (SOD), catalase (CAT), and enzymes of ascorbate–glutathione cycle ascorbate peroxidase (APX), mono-dehydro-ascorbate reductase (MDAR), dehydro-ascorbate reductase (DHAR) and glutathione reductase (GR) (Li et al., 2012, Logan et al., 2006). An investigation of wheat responses to WS (waterlogging and shading) stress applied at different grain –filling stages indicated that alternations in the expression of antioxidant enzyme-related and photosynthesis-involved genes might be responsible for bio-physiological modifications of wheat flag leaf caused by WS stress. Apart from the antioxidant enzymes, non-enzymatic antioxidants play a great role in response to over-accumulated ROS. For example, metallothionein2b (MT2b) was recognized as an important scavenger of H_2O_2 in rice (Wong et al., 2004). The decrease of MT2b enhanced epidermal cell death in leaves (Steffens and Sauter, 2009) as well as submergence-induced aerenchyma formation in the stem. These findings supported the view that altered ROS scavenging is highly efficient means to alter ROS levels. In epidermal cells above adventitious root primordia,

MT2b was shown to be down-regulated in response to ethylene or H_2O_2, indicating that reduced ROS scavenging through altered MT2b activity may be a general mechanism responsible for cell death regulation in rice (Steffens et al., 2011).

2.1.2 Morphological strategies for waterlogging stress tolerance

Plants experience three different stages of responses to the low-oxygen (Dennis et al., 2000). They are signal transduction (Stage I , 0~4 h), metabolic adjustment (Stage II, 4~24h) and morphological changes induced by the first two stages (Stage III, 24~48h) (Colmer and Pedersen, 2008, Steffens et al., 2011). The first stage is the determinant stage, which switches plants from normal metabolism pattern to low-oxygen – responsive pattern which contributes significantly to the survival of seedlings (Liu et al., 2012). Survival of plants under waterlogged conditions is largely attributable to by their ability to improve gas exchange between plants and environment as well as gas transport from aerial parts to hypogeal organs (Changdee et al., 2009). In Stage III , formation of aerenchyma (gas-filled spaces) in the root, stems or other shoot organs is observed (Liu et al., 2012). Aerenchyma are developed constitutively in many wetland plants as well as rice, which is essential for plants to tolerate frequent flooding, and is regarded as an efficient morphological adaptation to alleviate low oxygen stress (Colmer and Voesenek, 2009). Aerenchyma enhance gas exchange by reducing diffusion barrier to gas exchange imposed by cells (Steffens et al., 2011). Formation of new adventitious roots and the tolerance against radial oxygen loss (ROL) is another major adaptive trait of plants under waterlogging stress (Bacanamwo and Purcell, 1999, Mano and Omori, 2007). The structures of the root barriers are also capable of restricting entry of accumulated toxic compounds or metals during long-term waterlogging. Other traits such as lenticels at stem bases, and pressure driven flows along rhizomes in some species also play an important role in facilitating internal aeration (Kozlowski, 1984). Hyponastic growth of petioles of some rosette-type plants can prevent leaves from affecting by shallow floodwater stress (Colmer and Voesenek, 2009).

2.1.2.1 Aerenchyma formation

Three major pathways of aerenchyma formation are known in plants. Type I is lysigenous aerenchyma formed by programed cell death (PCD). Type I aerenchyma has been mainly studied in rice and maize roots, where cortex cells undergo PCD, resulting from exhaustion of sugars during waterlogging or submergence (Bailey-Serres and Voesenek, 2008).Type II is schizogenious aerenchyma formed by splitting of previously connected common cell wall. Type III is expansigenous aerenchyma (Bailey-Serres and Voesenek, 2008) or secondary aerenchyma (Shimamura et al., 2003), a white spongy tissue filled with large gas spaces. It is formed from living cell division or enlargement without cell separation or death. It is located in stems, hypocotyls, tap roots, adventitious roots and root nodules under flooded conditions (Shimamura et al., 2003). Cortical aerenchyma provides a pathway of low tolerance for transporting oxygen (Colmer and Voesenek, 2009). The gaseous hormone ethylene promotes the formation of aerenchyma by accumulation in plant organs during waterlogging or submergence due to the reduced diffusion rate (Rajhi et al., 2011, Steffens et al., 2011). This hypothesis was proved by applying exogenous ethylene which induced aerenchyma formation while ethylene inhibitors repressed the formation process (Drew et al., 1981, Jackson, 1985, Konings, 1982). However, it does not always require ethylene in aerenchyma formation, which was proved by lysigenous aerenchyma formation in the root of the wetland plant *Juncus effusus* (Mommer et al., 2006, Parlanti et al., 2011). It is suggested that disturbance to K^+ homeostasis-decreasing cytosolic K^+ pool, which caused the activation of PCD-related proteases, could be one ethylene-dependent pattern for lysigenous aerenchyma formation (Shabala, 2011). Aerenchyma formation could be also controlled by H_2O_2, indicating that ROS plays a key role in the regulation of diverse cell death processes in rice (Steffens et al., 2011). While ethylene controls aerenchyma formation in the fast-elongating '*Arborio Precoce'* variety, ROS accumulation plays an important role in a slow elongating variety (Parlanti et al., 2011). Aerenchyma formation involves multiple signal transduction pathways, in which Ca^{2+}, protein phosphorylation and G-protein are crucial signal components (He et al., 1996). However, molecular mechanisms underlying aerenchyma formation are not well understood.

2.1.2.2 Adventitious roots and ROL development

It is a common response in many wetland plant species to form adventitious root, which is another mechanism of adaption to long-term flooding. Adventitious roots can facilitate internal O_2 transport and even replace primary roots in some wetland species (Colmer and Pedersen, 2008, Jackson and Drew, 1984). Aquatic root growth as a response to flooding has been observed across a range of herbaceous crop species and numerous other non-crop species (Rich et al., 2012).Those chlorophyll-contained aquatic roots could even replace existing primary roots and became photosynthetically active (Rich et al., 2012). In addition, a barrier to ROL in the sub-apical regions of adventitious roots can be induced in many wetland plants (Armstrong et al., 2000, Colmer, 2003, Garthwaite et al., 2003). It is a physical response mainly resulted from secondary cell-wall deposits in outer hypodermal layer, which is a dominant mechanism of reducing ROL compared with the respiratory activity alternations in the hypodermal/epidermal layers (Garthwaite et al., 2008). An initial peak in ROL in roots of submerged rice resulted from initially high rates of net photosynthesis due to CO_2 accumulated during the dark while lower ROL in the roots was caused by sugar consumption overnight (Colmer and Pedersen, 2008, Waters et al., 1989). In contrast to wild relatives such as *Hordeum marinum*, barley and wheat do not form a barrier to ROL (McDonald et al., 2001). Moreover, though wheat, barley, and *H. marinum* all form adventitious roots containing aerenchyma, wheat and barley are much less tolerant to waterlogging than *H. marinum* (Garthwaite et al., 2006). It was also hypothesized that the formation of ROL structure in adventitious roots may affect water uptake (Garthwaite et al., 2006, Lee et al., 2005, Schreiber et al., 2005). However, by means of pressure-perfusion of solution through the large cortical air spaces in adventitious roots of rice, the measured hydraulic conductivity of external cell layers was 30-fold higher than overall root hydraulic conductivity, indicating that the 'apoplastic barriers' in the external cell layers of rice roots did not limit water uptake (Garthwaite et al., 2006, Ranathunge et al., 2003). Studies on deep-water rice under submergence showed that adventitious root emergence was regulated by multiple hormones, for example ethylene induced adventitious root directly, while auxin and cytokinin functioned indirectly by inducing ethylene synthesis (Lorbiecke and Sauter, 1999). In *Arabidopsis*, ethylene directed auxin to control root cell expansion and regulatory pathways in rice might be analogous to *Arabidopsis* (ZhiGuo et al., 2012).

2.1.2.3 Leaf gas films

For most marsh and water plants, leaf gas films were associated with the water repellence of submerged plants (Brewer and Smith, 1997, Neinhuis and Barthlott, 1997). A gas film can enlarge water-gas interface and may also maintain stomata to keep open when under water (Pedersen et al., 2009). Therefore, with leaf gas films uptake of CO_2 was enhanced for higher photosynthesis rate in light periods, as well O_2 exchange for respiration in dark periods. These functions of leaf gas films were presumably determined by micro- and nanostructures of the surface and wax crystals (Bhushan and Jung, 2006, Wagner et al., 2003). Removal of leaf gas films during light periods caused root pressure of O_2 (pO_2) to drop due to reduced underwater photosynthesis as CO_2 uptake reduced. During darkness, the removal of the gas films also caused a dramatic decrease in root pO_2 because of declined O_2 entry (Winkel et al., 2011).

2.1.2.4 LOES and LOQS Strategies

There are two main strategies for plants coping with complete submergence, which is the most stressful form of soil-flooding (Bailey-Serres and Voesenek, 2010). The first is an 'escape' strategy (LOES, low-oxygen escape syndrome) and the second is a 'quiescence' strategy (LOQS, low-oxygen quiescence syndrome) (Bailey-Serres and Voesenek, 2008, 2010). LOES–applied plants display enhanced shoot elongation in order to get access to the air when water level is low and the stress is long-term (>1-2 weeks). Some adaptive traits or mechanisms are specific for plants with LOES. These include: 1) retaining or developing anatomical structures or metabolic pathways that promotes gas exchange between plants and water environment, as well as within plant internal tissues. 2) Re-coordinating growth regulation and re-orientating energy flow for the establishment of shoot organs to grow above water. In contrast, LOQS-applied plants conserve the energy without shoot or leaf elongation when water level is deep and lasts only a short time (<1 week) (Bailey-Serres and Voesenek, 2008, Colmer and Voesenek, 2009). It was observed that the capacity of photosynthesis of newly developed leaves was retained in shoot-elongating plants during submergence, which resulted from decreased chlorophyll and chlorophyll fluorescence in older leaves (Sone et al., 2012). The shoot elongation caused by leaf elongation was faster than that by internodal elongation in *O. glaberrima*, although both leaf and internode elongation

were presumably stimulated by ethylene (Sakagami et al., 2009). The plants with LOES conserved some sucrose in the shoots during submergence which enabled them to recover (rapid re-growth and high photosynthesis rate) rapidly after de-submergence (Luo et al., 2011). Plants with LOQS not only use ATP economically, but also produce ATP in an O_2-deprived environment by regulation activities of some essential enzyme (Colmer and Voesenek, 2009). Interspecific diversity of these two strategies was found in both rice (Bailey-Serres et al., 2010, Fukao et al., 2006) and some species of the genus *Rumex* (Chen et al., 2011). For example, *R. palustris* responded to complete submergence with LOES while *R. acetose* responded with LOQS (Pierik et al., 2009). Within the genus *Lotus*, adult plants of *L. tenuis* were able to change from an escaping response to a quiescent strategy due to different water levels (Manzur et al., 2009). Seedling responses of three species *L. japonicus*, *L. corniculatus* and *L. tenuis* to complete submergence accompanied by recovery phases showed interspecific difference at early growth stage (Striker et al., 2012). In rice, quiescence strategy is employed by some deep-water or floating rice cultivars to perform the tolerance (Perata and Voesenek, 2007), but "quiescence strategy" is not necessarily responsible for tolerance to low oxygen stress for plants at the seedling stage (Ismail et al., 2009, Vu et al., 2010)

2.1.3 Metabolism and signalling network in response to waterlogging stress

In rice, the promotion or inhibition of root growth is controlled by multiple hormones and metabolisms (Bailey-Serres et al., 2012). Ethylene plays a crucial role in plant reaction to waterlogging. Flooding -induced ethylene supresses ABA production and the decrease of ABA was subsequently followed by an increased GA responsiveness (Saika et al., 2007). It is found that the highly submergence-inducible *SUB1A* gene in rice encodes ethylene response factors (ERFs) and submergence tolerance is strongly correlated with the presence of the *Sub1A-1* allele (Fukao et al., 2011, Perata and Voesenek, 2007). *Sub1A-1* transgenic genotypes have higher accumulation of GA-signalling repressors SLR1 and SLRL1, which decreases GA responsiveness, thus enhances starch breakdown (Fukao and Bailey-Serres, 2008, Sun, 2011). Other evidence derived from GA insensitivity of *Sub1A-1* transgenic plants and enhanced shoots elongation in M202 (*Sub1*) by ethylene precursor provide support for the assumption that low oxygen quiescence strategy is facilitated by ethylene driven

expression of *Sub1A-1* and decreased GA responses (Barding et al., 2012, Fukao and Bailey-Serres, 2008).

Apart from ethylene, low oxygen is another direct signal of plant reacting to flooding stresses. It is suggested that there are mainly two low-oxygen sensing mechanisms in plants, direct and indirect sensing mechanisms. Direct sensing mechanism includes certain transcriptional factors (TFs) and hypoxia-inducible, oxygen-binding plant hemoglobins. One key transcriptional regulator is hypoxia-inducible factor α (HIF α) which induces many hypoxia-responsive genes (Sasidharan and Mustroph, 2011). In *Arabidopsis*, group VII ERF families were confirmed to be involved in direct sensing mechanism. They also regulated plant responses to drought, salt or osmotic stresses, which suggested the presence of a complex regulatory network. Indirect sensing mechanism is mediated by signals or variations in the status or concentrations of metabolites, such as adenylates, carbohydrates, and pyruvate, and localized cellular changes in pH, $Ca^{2+,}$ ROS (H_2O_2, NO), NADH/NAD^+ ratios, or the energy status (ATP levels). The changes in energy levels could be signals in many protein kinases-regulated physiological processes, such as the SnRK1 (KIN10/11 in *Arabidopsis*)-regulated carbon management (Baena-Gonzalez, 2010, Bailey-Serres et al., 2012), calcineurin B-like interacting protein kinase (CIPK15)-involved anaerobic germination of rice seedlings (Lee et al., 2009). In maize roots, about 20 anaerobic proteins (ANPs) were induced by low oxygen treatment. Most of them were recognized as enzymes involved in sugar phosphate metabolism. These include alcohol dehydrogenase, aldolase, enolase, glucose phosphate isomerase, glyceraldehyde-3-phosphatedehydrogenase, pyruvate decarboxylase (PDC) and sucrose synthase. Those enzymes could also be recognized as indirect sensors (Zou et al., 2010).

As a signalling molecule, ROS is able to accumulate rapidly during stresses. This internal change could be assumed as a clue to understand interaction pattern between submergence tolerance and tolerance to other stresses, such as re-oxygenation or drought (Blokhina and Fagerstedt, 2010, Miller et al., 2010). *Sub1 A* has an important role in transferring ethylene signal by negatively affecting ROS production. It was also demonstrated that the synthesis of antioxidant enzymes was more active in tolerant genotype M202 (*Sub1*) during submergence (Jung et al., 2010, Mustroph et al., 2010) and contributed to less oxidative injury and faster recovery upon de-submergence than

M202 (Fukao et al., 2011). In addition, *Sub1A* also promotes ABA responses caused by dehydration, which include the reduction of water loss, triggered LEA proteins production and better recovery of tiller growth after severe drought stress (Fukao et al., 2011). Altogether, it is proposed that *Sub1A* gene is responsible for regulating tolerance to multiple abiotic stresses by altering its transcriptional and translational levels (Fukao et al., 2011).

2.1.4 Exploration and utilization of barley genetic germplasm with high waterlogging tolerance

2.1.4.1 Identification of waterlogging tolerance genes in crops

For barley, earlier waterlogging tolerance studies were conducted in glasshouse-based pot experiments. Leaf chlorosis, biomass reduction and survival were evaluated for waterlogging tolerance (Table1). Twenty QTL for waterlogging tolerance related traits were found in the two barley double haploid (DH) populations TX9425 × Franklin and Yerong × Franklin. Several of these QTLs were validated through replication of experiments across seasons or by co-location across populations (Li et al., 2008). Components of grain yield **(Table 2.1)**, such as grains per spike, spikes per plant, kernel weight and spike length were also chosen as indicators of waterlogging tolerance screening. Major QTL related to those traits were located at linkage group 2H (Xue et al., 2010). Following the development of more reliable screening facilities that more closely resemble field conditions, more accurate QTLs were identified. A novel score system (combined score of leaf chlorosis and plant survival rate under waterlogged conditions) was employed. From three DH populations (TX9425 × Naso Nijo, Yerong × Franklin and YYXT × Franklin), eight QTL were identified (Xu et al., 2012, Zhou, 2011, Zhou et al., 2012) **(Figure 2.1)**, most of which were located at linkage group 2H and 4H. Tolerance genes from Yerong and YYXT were mostly located in a similar position while that from TX9425 were at different positions. Moreover, one QTL (4H) for aerenchyma formation from population YYXT × Franklin was located at a similar position to a QTL (4H) for waterlogging tolerance inferred from population Yerong × Franklin, confirming the relationship between aerenchyma formation and waterlogging tolerance (Zhou et al., 2012).

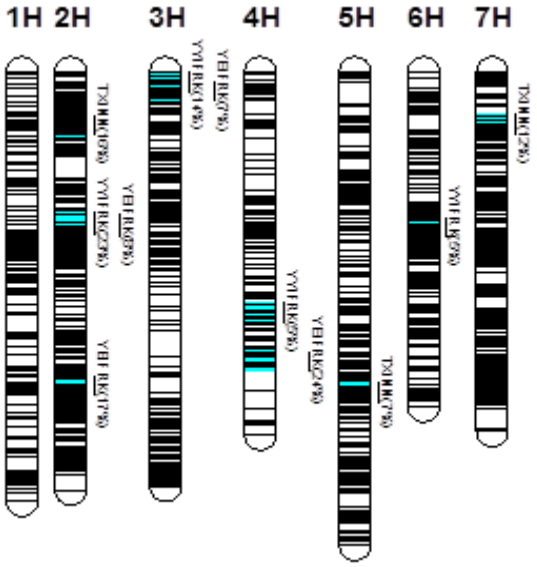

Figure 2.1 QTL identified for waterlogging tolerance in barley. TX/NN: cross of TX9425 and Naso Nijo (Xu et al., 2012); YE/FRK: cross of Yerong and Franklin (Zhou, 2011); YY/FRK: cross of YuYaoXiangTian (YYXT) and Franklin (Zhou et al., 2012). The numbers in bracket are percentages of phenotypic variation determined by the

Table 2.1 Summary of QTL that influence flooding/waterlogging tolerance

phenotypic traits	crops	parents of population[a] (size)	population type[b]	chromosome	references
adventitious root formation	maize (*Zea mays* L.)	B64 × **teosinte** (*Z. mays ssp. Huehuetenangensis*) (94)	F_2	4, 5, 8	(Mano et al., 2005)
	rice (*Oryza Sativa* L.)	**IR1552**(*O. indica*) × Azucena (*O. japonica*) (96)	RIL	3, 4, 9	(Zheng et al., 2003)
flooding tolerance index (relative seed weight)	soybean (*Glycine max* L.)	**Misuzudaizu** × Moshidou Gong 503 (156)	RIL	LG C2 (largest QTL)	(Githiri et al., 2006)
germination rate	rice	**Khao Hlan On** (*O. japonica*) × IR64 (423)	BC_2F_2	1, 3, 9	(Angaji et al., 2010)
	rice	**IR72** × **Madabaru**[c] (466)	$F_{2:3}$	1, 2, 9, 12	(Septiningsih et al., 2012)
	rice	**Kinmaze** (O. *japonica*)× DV85 (*O. indica*) (81)	RIL	1, 2, 5, 7	(Jiang et al., 2004)
	soybean	Nannong 1138-2 × **Kefeng No.1** (151)	RIL	LGA1, LGD1a, LG G	(Wang et al., 2008)
grains per spike	barley (*Hordeum vulgare* L.)	**Yerong** × Franklin (156)	DH	2H, 5H, 7H	(Xue et al., 2010)
grain yield	barley	**Yerong** × Franklin (156)	DH	2H, 7H	(Xue et al., 2010)
internode elongation	rice	**C9285**(*O. indica*) × T65 (94) **W0120** (*O. rufipogon*)× T65 (94)	F_2	12	(Hattori et al., 2007, Hattori et al., 2008)
kennel weight	barley	**Yerong** × Franklin (156)	DH	2H	(Xue et al., 2010)
leaf chlorosis	barley	Franklin × **TX9425** (92)	DH	1H, 2H, 3H, 7H	(Li et al., 2008)
	barley	**Yerong** × Franklin (177)	DH	1H, 2H, 3H, 4H, 5H, 7H	(Li et al., 2008)
plant biomass reduction	barley	Franklin × **TX9425** (92)	DH	4H	(Li et al., 2008)
	barley	**Yerong** × Franklin (177)	DH	4H	(Li et al., 2008)
plant height	barley	**Yerong** × Franklin (177)	DH	6H	(Xue et al., 2010)
	maize	**HZ32** × K12 (228)	F_2	1, 4, 7, 10	(Qiu et al., 2007)

Table 2.1 (Continued)

phenotypic traits	crops	parents of population[a] (size)	population type[b]	chromosome	references
plant survival	barley	**Yerong** × Franklin (177)	DH	2H, 5H	(Li et al., 2008)
	barley	Franklin × **TX9425** (92)	DH	2H	(Li et al., 2008)
root aerenchyma formation	maize	B73 × **teosinte (*Z. luxurians*)** (195)	F_2	2, 5, 9, 10	(Mano et al., 2008)
	maize	Mi29 × **teosinte** (123)	BC_4F_1	1	(Mano and Omori, 2009)
root dry weight	maize	**HZ32** × K12(228)	F_2	1, 6, 9	(Qiu et al., 2007)
root length	maize	**HZ32** × K12(228)	F_2	7	(Qiu et al., 2007)
seedling vigor	rice	Lemont(O. *japonica*) × **Teqing** (*O. indica*) (282)	RIL	1, 3, 5, 10	(Zhou et al., 2007)
shoot dry weight	maize	**HZ32** × K12(228)	F_2	3, 4, 6, 9	(Qiu et al., 2007)
spike length	barley	**Yerong** × Franklin (156)	DH	2H, 3H	(Xue et al., 2010)
spikes per plant	barley	**Yerong** × Franklin (156)	DH	2H, 6H, 7H	(Xue et al., 2010)
stem elongation	rice	Patnai 23 × **Goai (a floating parent)** (186)	F2	12	(Nemoto et al., 2004)
total dry weight	maize	**HZ32** × K12(228)	F_2	3, 4, 9	(Qiu et al., 2007)
Waterlogging score (combined leaf chlorosis and plant survival rates)	barley	**Yerong** × Franklin (177)	DH	2H, 3H, 4H	(Zhou, 2011)
	barley	**TX9425** × Naso Nijo (188)	DH	2H, 4H, 5H, 7H	(Xu et al., 2012)
	barley	Franklin × **YYXT** (172)	DH	2H, 3H, 4H, 6H	(Zhou et al., 2012)
	soybean	A5403 × **Archer** (103)	RIL	LG A1, LG F, LG N	(Cornelious et al., 2005)
	soybean	P9641× **Archer**(67)	RIL	LG A1, LG F, LG N	(Cornelious et al., 2005)
waterlogging tolerance index (combined plant height and number of leaves at the end of waterlogging)	soybean	**Su88-M21** × NJRISX (175)	RIL(156)	LG L2	(Sun et al., 2010)

[a]Parents which are more tolerant are marked in bold; [b] RIL represents for recombinant inbred lines, DH for double haploid population, NIL for near-isogenic lines;
[c] Both IR72 and Madabaru are moderately tolerant

In rice and other crops, various morphological traits were used to identify QTL associated with waterlogging tolerance **(Table 2.1)**. An F2:3 population developed from IR72 and Madabaru, both moderately tolerant varieties, was investigated to identify novel QTL that were non-allelic to the Sub1 alleles. Using survival rates as the evaluation traits and SSR (simple sequence repeats) maker and indel markers derived molecular data, three QTL the same as identified in IR72 were identified, which were located in chromosomes 1, 2 and 12. The results further suggested that an alternative pathway may be present in this variety that is not related to the regulatory pathway mediated by the *Sub1A* gene. These novel QTL can be used to develop novel varieties with enhanced level of submergence tolerance for flood-prone areas (Septiningsih et al., 2012). To facilitate the direct seedling of rice in both rainfed and irrigated areas, screening of 329 BC2F2 lines were conducted and identified five putative QTL explaining 17.9 to 33.5% of the phenotypic variation (seed survival and germination rate) at seedling stage, of which three were not located at the same chromosome as *Sub1A* locus (Chromosome 9) (Angaji et al., 2010). Among the target traits, aerenchyma formation and adventitious root formation are most directly related to a plant's ability to adapt to low-oxygen stresses. However, it seems that aerenchyma formation is correlated with at least four QTL on three different chromosomes in maize (Mano and Omori, 2007). Moreover, these QTL explained less than 50% of plants' phenotypic variation under waterlogged conditions (Shabala, 2011). Therefore, accurate mapping for waterlogging-tolerance genes is demanded. Different molecular marker methods were employed depending on the level of polymorphism and detection sensitivity in different mapping populations or lines (Li and Quiros, 2001).

2.1.4.2 Identification and evaluation of waterlogging tolerant barley genotypes

Evaluation of waterlogging tolerance is complicated as timing, duration and severity of stress could vary greatly (Setter and Waters, 2003). Moreover, genetic difference in response to waterlogging stress is found in several species, such as maize and wheat (Pang et al., 2004). It is found that barley displayed various tolerance mechanisms when exposed to intermittent waterlogging, such as adventitious root development and aerenchyma formation and selection of effective index and screening time period is crucial for screening tolerant genotypes (Bailey-Serres et al., 2012).

To simulate the waterlogging stress occurring at germination stage, stagnant deionised water was used to screen a collection of >3400 world barley germplasm. It was found that 1) genotypes with low viability (40–90%) have an even low tolerance to waterlogging. 2) Low temperature (0–5 °C) treatment to seeds can totally eliminate or reduce adverse effects of waterlogging stress compared with optimal temperature (25 °C) treatment to seeds after 4-day waterlogging stress. 3) Barley varieties differ significantly in waterlogging tolerance at the germination stage. Meanwhile, the tolerance to waterlogging stress was found correlated with varietal geographic distribution, for example, varieties from East Asia were more tolerant than those from West Asia (Setter and Waters, 2003). This phenomenon could be explained by the natural or artificial selection for tolerance traits related with the climate variations. When waterlogging stress is imposed at the whole-plant stage, yield of plants is the main index for waterlogging tolerance evaluation (Setter and Waters, 2003).

As barley genotypes respond to waterlogging stress differently under varying environmental or experimental conditions with more than one limiting factor, it is reasonable to combine several adaptive traits to evaluate the levels of waterlogging tolerance under specific environment condition (Pang et al., 2004, Zhou et al., 2012). For example, chlorophyll fluorescence technique (specifically, the Fv/Fm parameter) is regarded as promising and reliable for quick screening of waterlogging tolerance (Pang et al., 2004). It is also shown that recovering ability of root system from transient waterlogging or hypoxia is crucial for waterlogging tolerance. Those survival traits include the least pronounced reduction of photosynthetic rate, chlorophyll content and fluorescence, and a rapid recovery after drainage for these characteristics (Pang et al., 2004). Moreover, maintenance of adequate nutrition for least loss of plant yield and biomass is related to approaches of crop management for waterlogging tolerance (Setter and Waters, 2003). On the other hand, most of these trait measurements, for example, net O_2 and ion fluxes from the root surface and net CO_2 assimilation are applicable for screening a small amount of lines. It is not practical for breeders to use these indices to screen thousands of lines during a limited time period. Hence development of molecular markers associated with barley waterlogging tolerance and marker-assisted selection could be applied for waterlogging screening (Zhou et al., 2012)

2.1.4.3 Development of new waterlogging-tolerant barley genotypes

Barley suffers yield loss in waterlogged soils, while the solution of drainage work for alleviating adverse effects caused by waterlogging may not be justified by improved crop returns. Therefore development of waterlogging-tolerant barley varieties may be a sustainable solution (Pang et al., 2004). For barley, it is plausible to improve their tolerance to waterlogging by hybridization with their wild relatives such as *H. marinum*, a wetland species highly tolerant to waterlogged and saline environment. Improved root aeration, including stronger formation of root barrier for ROL and higher root porosity, has been observed in *H. marinum*-wheat amphiploids hybrid (Bailey-Serres et al., 2012, Malik et al., 2011), which provides a promising breeding prospective for waterlogging tolerance in wheat and barley. Meanwhile, based on the progress of QTL mapping, development of near-isogenic lines (NILs) is necessary for targeting the waterlogging tolerant gene accurately, which provides another promising way of variety improvement. NILs can eliminate the noise caused by additional genes or population structure and resolve the mapping position of QTL, as fixed genetic background for affecting phenotypic assessments of quantitative traits is needed (Chen et al., 2012a). NIL-derived populations, segregating primarily for a targeted locus, allow the conversion of a quantitative trait into a Mendelian factor, making the accurate location of a QTL possible (Ma et al., 2012). They have been used intensively for investigating the effects of various genes of interest, for example the *Fusarium* head blight and crown rot tolerance gene in wheat (Ma et al., 2012, Miedaner and Voss, 2008).

2.1.4.4 Strategies of enhancing waterlogging tolerance in other crops

In rice, *Sub1* gene cluster has a crucial role in quiescence reaction to a short-term flooding (Septiningsih et al., 2012). Introgression of *Sub1* QTL into 'Swarna' greatly improved plant survival rate and grain yield under submergence. Six submergence-tolerant mega varieties with *Sub1* locus were developed and released to Asian countries for farmland production, such as IR 64- *Sub1* and BR11- *Sub1* (Iftekharuddaula et al., 2011, Neeraja et al., 2007, Septiningsih et al., 2009). Varieties or sources with enhanced tolerance to long-duration submergence are needed (Septiningsih et al., 2012), especially those combined with tolerance to other environmental factors such as water turbidity, water temperature and light density (Xu et al., 2006). However, breeding and

adoption of new varieties are time-consuming. Therefore, introgression of tolerant genes with molecular techniques is more efficient compared with the traditional breeding methods. For example, the method of marker assisted backcrossing (MABC) was used efficiently in incorporating tolerant genes into popular varieties (Cuc et al., 2012). MABC is an effective method to introduce a single locus controlling a trait of interest without confounding the genetic background of the recurrent parent. This approach has been used with great success for 'enhancing' cereals for traits (Collard and Mackill, 2008). They include bacterial blight tolerance gene *Xa21* in rice (Chen et al., 2000), powdery mildew tolerance gene *Pm* in wheat (Zhou et al., 2005), submergence tolerance *Sub1* (Neeraja et al., 2007, Septiningsih et al., 2009), the yellow dwarf virus tolerance gene *Yd2* (Jefferies et al., 2003) and leaf rust tolerance gene *Rphq6* in barley (Berloo et al., 2001). The other strategy to identify limiting factors of waterlogging tolerance is direct genetic engineering, which includes two approaches: 1) down-regulating or up-regulating the expression of single candidate genes, 2) over-expression or down-expression of relative transcription factors (Dennis et al., 2000). Both of two approaches are efficient in converting targeted traits as well as evaluating the interaction of diverse mechanisms of tolerance to waterlogging. They are also effective in triggering the longer term adaptation response to low oxygen stress (Bidhan et al., 2011). For example, it was showed that plants with the transferred chimeric SAG12-*ipt* gene displayed higher-level of tolerance to waterlogging than wild-type plants (Zhang et al., 2000). The improved tolerance may be explained by accelerated function of anti-oxidative system in transgenic plants. It is also suggested that higher endogenous cytokinins in the transgenic plants may facilitate plant to be more adaptive to low oxygen conditions (Tereshonok et al., 2011). Other successful transgenic genotypes developed include: 1) transgenic rice with the *pdc1* gene, which improved the submergence tolerance and enhanced the activity of PDC. 2) transgenic cotton with *adh* gene which displayed more efficient ethanol fermentation to reduce the ethanol caused injury (Dennis et al., 2000).

2.2 Aluminium toxicity

As the most abundant metal in the earth's crust, aluminium (Al) can be toxic when solubilised in soils at low pH (<5.0) to form phytotoxic Al^{3+} (Hoekenga et al., 2003, Wang et al., 2006). The initial adverse effects of Al toxicity are inhibiting root elongation and causing structural damages to the root tissues, which are followed by limiting water uptake and nutrients absorption (Kochian, 1995, Melakeberhan et al., 2001, Parker, 1995). Al can begin to inhibit root growth of wheat within minutes or hours in simple hydroponic solutions (Ryan et al., 1993) affecting both root cell elongation and root cell division (Amenos et al., 2009). Large swollen cortical cells were found in wheat and maize root tips shortly after Al treatment (Ciamporova, 2002, Zelinova et al., 2011). Exposure to Al toxicity causes accumulation of cell wall polysaccharides and lignin, resulting in the typical thick and rigid cell wall in wheat (Tabuchi and Matsumoto, 2001) and rice (Yang et al., 2008). These Al-dependent changes in the cell wall are involved in Al-induced reduction of cell expansibility, which may be the main cause of the Al inhibition of root elongation in short-term experiments (Ma et al., 2004). Longer exposures result in thickened roots, damaged root cap, and lesions in the epidermal and cortical tissues near the apices (Foy, 1984, Ryan et al., 1993, Zeng et al., 2013). Many mechanisms can be involved in Al toxicity. They include inhibition of cell division, disjunction of cell wall, inhibition of ion fluxes, disruption of plasma membrane integrity, failure in calcium homeostasis, inhibition of signal transduction pathways, alteration in cytoskeleton structure and production of oxygen radicals (Matsumoto, 2000, Zelinova et al., 2011, Zheng and Yang, 2005).

2.2.1 Deleterious effects of Al on plant metabolisms

2.2.1.1 Oxidative stress

Al has a strong affinity to bio-membranes, and the Al accumulation causes changes in the membrane structure and function, affecting the permeability of liposomes and packaging of fatty acids of the plasma membranes (Inostroza-Blancheteau et al., 2012, Oteiza, 1994). Internal ROS production can be triggered by Al, which can be accompanied by the respiration inhibition and ATP depletion. These are consistent with

the inhibition of cell growth (tobacco cells) and the inhibition of root elongation (pea roots) when plants are exposed to Al toxicity (Yamamoto et al., 2002). The oxidative stress produced by Al toxicity causes an increase in the production of ROS such as superoxide radicals (O_2^-), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2) (Guo et al., 2007). The ROS may affect biological macromolecules, such as unsaturated fatty acids in the cell membrane, causing peroxidation of membrane lipids, which in turn may lead to severe cellular damage (Basu et al., 2001, Inostroza-Blancheteau et al., 2012, Kochhar and Kochhar, 2005, Yamamoto et al., 2002). H_2O_2 could be a substrate for cell wall peroxidase and can accumulate to an adverse level when ascorbate production is inhibited. It was shown that cell wall loosening, which is essential for cell elongation growth, can be induced by ·OH produced in the cell walls in a peroxidase-catalysed reaction from O_2^- and H_2O_2 (Liszkay et al., 2004).

The destructive effects of Al-induced ROS in plants are counteracted through antioxidant mechanisms (Basu et al., 1999). When these mechanisms are saturated, Al induces damage in cells and tissues, increasing the level of lipid peroxidation, which alters the activities of antioxidant enzymes (Basu et al., 2001). Al tolerance in plants is reliant on antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), as well as non-enzymatic molecules of low molecular weight, such as ascorbic acid (AsA), reduced glutathione (GSH) and phenol like compounds such as tocopherols (α-tocopherol), flavonoids, carotenoids (β-carotene), and uric acid (Dabrowska et al., 2007, Guo et al., 2007, Kochhar and Kochhar, 2005, Yoo et al., 2007). The suppression of peroxidase activity can maintain normal H_2O_2 levels required for cell wall remodelling, a process also associated with Al tolerance (Córdoba-Pedregosa et al., 2005, Houde and Diallo, 2008, Souza et al., 2002). Al toxicity induced genes that code for enzymes SOD, POD and glutathione S-transferase (GST), and also resulted in an increase in the SOD (Cakmak and Horst, 1991, Qin et al., 2010) and APX activities (Cakmak and Horst, 1991, Dipierro et al., 2005). Additionally, these genes were also induced by oxidative stress (Inostroza-Blancheteau et al., 2012, Richards et al., 1998). For example, the adverse effects caused by Al toxicity in *Brassica napus* can be alleviated by over-expression of a manganese superoxide dismutase which may be explained by the improved ability to deal with the oxidative stress caused by Al^{3+} (Basu et al., 2001, Delhaize et al., 2009).

The plant hormone auxin plays an important role in not only the responses to oxidative stress (Krishnamurthy and Rathinasabapathi, 2013, Tamas et al., 2012), but also the distribution of Al in cells (Zhu et al., 2013). For example, auxin might be the connecting link that regulates the level of ROS and direct the role of ROS in oxidative stress (Krishnamurthy and Rathinasabapathi, 2013). A recent study found that compared with the wild-type ecotype Columbia, an auxin-overproducing mutant, *yucca*, had significantly reduced cell wall Al and increased symplastic Al content, suggesting that auxin may regulate Al distribution in root cells (Zhu et al., 2013). Acid-growth theory indicates that auxin can activate plasma membrane (PM) H^+-ATPase and facilitate H^+ efflux into the cell wall compartment, thus softening the cell wall and initiating extension growth (Grebe, 2005, Hager, 2003, Wu et al., 2014).

The role of Al-induced ROS production as a signal leading to enhanced Al tolerance has been identified in sorghum. The *SbMATE* gene encoding an Al-activated citrate transporter, was responsible for the major sorghum Al tolerance locus. It was found that the long lag between Al exposure, root citrate exudation and induction of *SbMATE* expression could be due to a requirement for the cellular signal of ROS to accumulate above a threshold level (Liu et al., 2014, Magalhaes et al., 2007). It is found that Al induced the greatest cell damage and generation of ROS specifically in the root distal-transition-zone of roots (Sivaguru et al., 2013). The co-localization of the highest Al-induced ROS production in the very same root region where *SbMATE* expression is the highest is interesting. It could simply be that the plant is expressing this citrate transporter where the Al toxicity is the greatest. Alternatively, these associations do lend themselves to the speculation that ROS production might be associated with Al signalling. Hence, the high ROS levels in response to Al might serve as a monitoring signal triggering events that lead to increased SbMATE protein abundance and protection of the root cells in this region from Al toxicity (Liu et al., 2014). Even though it has been widely reported that Al induces ROS and increases oxidative stress in plants (Panda et al., 2009, Yamamoto et al., 2002), recent findings show that ROS-induced stress may be due to acidic conditions (low pH) rather than to Al toxicity. Nevertheless, it must be taken into account that it is not possible to have Al toxicity without acidic conditions (Inostroza-Blancheteau et al., 2012).

2.2.1.2 Al disturbance to calcium homeostasis and signalling

It has been shown that Ca^{2+} plays a crucial role in root elongation and determining the structural rigidity of the cell wall (Hepler, 2005, Wang et al., 2006). Low concentration of external Ca^{2+} could make the cell wall more pliable and easily ruptured, while high concentrations could make cell walls more rigid and less plastic for remodelling (Hepler, 2005, Tagawa and Bonner, 1957). External Ca^{2+} treatment with concentrations between 0.1 to 1.0 mM was demonstrated to be necessary for integrity and functional permeability of plasma membrane (Kinraide et al., 1985). Based on these observations, increase of calcium concentrations in solution of the root rhizosphere, could be an efficient solution for Al toxicity (Wang et al., 2006). Al has been reported to induce either increases (Rengel, 1992) or decreases (Jones et al., 1998) in cytosolic Ca^{2+} depending on the experimental system studied. These changes in cytosolic Ca^{2+} have generally been suggested to be a cause of Al toxicity. However, given the mounting and clear evidence for Al induction/ activation of Al tolerance processes, it is not unreasonable to speculate that Al induced changes in cytosolic Ca^{2+} could be part of signalling and/or regulatory pathways leading to enhanced tolerance (Liu et al., 2014). The role of Ca^{2+} as a second messenger for participation in such signalling /regulatory pathways is well documented when Al induced increases in ROS in plant roots (Jones et al., 2006). Again as with the Al-induced alterations in the cytosolic Ca^{2+}, Al-induced increases in ROS production have usually been associated with root cell damage caused by Al toxicity (Jones et al., 2006, Yamamoto et al., 2003).

2.2.1.3 Impacts of Al on phosphorus, boron, magnesium and nitrogen nutrition

Al toxicity and phosphorus (P) deficiency often coexist in most common upland soils (Zheng, 2010). Al-P interactions in plants were associated with plant performance in acid soils, instead of the interaction between Al and P in soil solutions. As Al toxicity in acid soils prevents root development of many crops, the reduction of the root system makes crops more sensitive to other abiotic stresses such as water and nutrient stress, thus reduces the ability of crop plants to acquire P from the soil and reduce the yield (Hairiah et al., 1995). Presumably, sufficient P nutrition could promote the growth of plants and improve tolerance to other abiotic stresses. Previous studies found that P addition could alleviate Al toxicity in plants (Gaume et al., 2001, Liao et al., 2006,

Nakagawa et al., 2003, Silva et al., 2001, Tan and Keltjens, 1990). This alleviation effect is likely based on two possible mechanisms: direct interactions of Al-P precipitation in soil solution and plants (on the root surface, in root cell walls or within root cells) (Taylor, 1991, Zheng et al., 2005) and indirect effects, such as, alleviation of Al toxicity by P application via improving the root morphology and facilitating nutrient uptake or by secreting special root exudates (Liao et al., 2006). There are also reports claiming that Al-resistant cultivars could use P more effectively than the Al-sensitive cultivars in both rice (Sivaguru and Paliwal, 1993) and sorghum (Ramirez and Lopez, 2000). Delhaize et al. (Delhaize et al., 2009) found that transgenic barley expressing *TaALMT1* (Al tolerance gene of wheat) was more efficient in P uptake on acid soils than a non-transformed sibling line (Chen et al., 2012b).

As a structural component of the cell wall (CW), boron (B) poses its primary influence in the CW and at the plasma membrane-CW interface. The root CW plays a crucial role in the expression of Al toxicity in sensitive species and many studies have shown that CW is the main compartment of Al accumulation (Horst et al., 2010, Yang et al., 2011). A high sensitivity to Al toxicity would correspond to a high proportion of un-methylated pectate residues in the root tip CW (Horst et al., 2010). As both Al and B primarily target plant CW, interactions between these elements are to be expected. Within plants the main Al species probably is $Al(OH)_3$ (Kochian, 1995), which has structural similarity to $B(OH)_3$. Alleviation of Al-induced inhibition of root growth by supra-optimal concentrations of B has been observed in Al-sensitive plants (Corrales et al., 2008, Jiang et al., 2009, Stass et al., 2007, Yang et al., 2004, Yu et al., 2009). Interactions between Al and B at the level of the carbohydrate metabolism and the antioxidant system have been investigated (Hajiboland et al., 2015, Jiang et al., 2009, Ruiz et al., 2006).

It is demonstrated that magnesium (Mg) could increase plant tolerance to Al toxicity in sorghum, wheat, soybean and rice (Chen and Ma, 2013). As Mg^{2+} has similar hydrated radius with soluble Al, it could compete for the same cellular components or transporter as Al at root cell wall and plasma membrane to alleviate the adverse effects (Bose et al., 2011). The external Mg^{2+} supply could also increase the ionic strength of the solutions and reduce the Al saturation at the apoplastic exchange sites (Grauer and Horst, 1992, Noble et al., 1988). High concentration of Mg^{2+} at millimolar levels is found to be

required for alleviating Al toxicity in wheat and rice (Ryan et al., 1997, Watanabe et al., 2005). On the other hand, Mg-alleviated Al toxicity can be attributed to enhancement in secretion of citrate acid, which can combine with Al and form non-toxic Al-citrate complex. This phenomenon has been found in soybean and rice bean, although the exact mechanism of increase in organic acid secretion was not clear (Chen et al., 2012c, Silva et al., 2001, Yang et al., 2007). It has been found that the up-regulation of one Mg^{2+} transporter gene *OsMTG1* is required for conferring Al tolerance in rice (Chen et al., 2012c). However, the expression level of all members of *AtMTG* gene family in *Arabidopsis* cannot be enhanced by Al (Zhao et al., 2009). These results seem to suggest that, as the most Al-tolerant small cereals, rice may have a distinct strategy to cope with Al toxicity (Chen et al., 2012c).

Due to weak nitrification capacity and the application of ammonium fertilizers on these soils, the predominant inorganic for of nitrogen (N) in acid soil is ammonium (NH_4^+) (De Boer and Kowalchuk, 2001, Guo et al., 2010, Schroder et al., 2011). Uptake of NH_4^+ by roots is known to be accompanied with release of H^+, resulting in pH decrease in the rhizosphere (Schubert and Yan, 1997). Since Al solubility increases with decreasing soil pH, it is not unreasonable to speculate that the presence of NH^{4+} increases the sensitivity of plants to Al toxicity. An alleviative effect of NH_4^+ on Al toxicity has been observed in both *japonica* and *indica* cultivars differing in Al sensitivity in rice (Zhao et al., 2013). This effect of NH_4^+ is associated with decreased Al accumulation in the root tips (Zhao et al., 2009). Furthermore, a significant correlation between N preference and Al tolerance has recently been observed in 30 rice cultivars (Zhao et al., 2013). The alleviative effect of NH_4^+ on Al toxicity has been partly attributed to a direct competition for root binding sites between Al^{3+} and NH_4^+ in *Lespedeza bicolor* (Zhao and Shen, 2013). In roots of both Al-sensitive and Al-tolerant rice cultivars, it was found that NH_4^+-reduced accumulation of Al is a consequence of altered cell wall properties. The suppression of Al accumulation was triggered by pH decrease due to NH_4^+ uptake, rather than direct competition for the cell wall binding sites between Al^{3+} and NH_4^+ (Wang et al., 2015).

2.2.2 Mechanisms of Al tolerance

Differences in tolerance to acid soil exists among different species and genotypes of the same species with two main mechanisms being reported to control Al tolerance (Wang et al., 2006). The external tolerance mechanism also referred as exclusion mechanism has been intensely studied, which is explained by Al-triggered or non-Al-triggered release of organic anions to chelate toxic Al and limit Al uptake (Delhaize et al., 2012, Maron et al., 2013, Ryan et al., 2001). Two gene families, *MATE* (multidrug and toxic compound extrusion) and *ALMT* (aluminium -activated malate transporter) genes, were found to be responsible for the external mechanism, both encoding membrane transporter proteins and facilitating the organic anion efflux (Delhaize et al., 2012). For internal mechanism, the detoxification process involves Al^{3+} relocation and chelating with organic acids (citrate and oxalate) (Garcia-Oliveira et al., 2013).

Barley is among the most Al-sensitive cereal crops and genotypic variation exists within this species (Zeng et al., 2013). Al-tolerance mainly relies on the release of citrate from root apices in barley (Ma et al., 2004, Ma et al., 2001, Ryan et al., 2001), which is controlled by HvMATE transporter. *HvMATE* gene (also referred as *HvAACT1*) has been detected in most tolerant barley genotypes (Bian et al., 2013, Raman et al., 2002, Tang et al., 2000, Wang et al., 2006, Wang et al., 2007). This gene is located on a same region as the *Alp* locus in chromosome 4HL and has been designated as the major gene conferring Al tolerance (Furukawa et al., 2007, Ma et al., 2004, Wang et al., 2007). Expression of *HvAACT1*can enhance Al-activated citrate efflux and increase Al tolerance (Zeng et al., 2013). However, through transgenic study, it appeared that the increase of Al tolerance by *HvAACT1* over-expression in barley was not as effective as Al-tolerance gene *TaALMT1* in wheat (Zeng et al., 2013). Al tolerance is associated with the polymorphisms of MATE and ALMT genes (Fujii et al., 2012). Transposable elements are capable of altering the *HvAACT1*gene expression (Delhaize et al., 2012). As it is well known that *HvAACT1* is constitutively expressed in root epidermal cells, a 1023-bp insertion in the 5' untranslated region (UTR) can alter its original expression location which is sensitive to Al toxicity to mature zone (Furukawa et al., 2007). Moreover, this insertion can significantly promote the expression of *HvAACT1* in tolerant barley genotypes (Fujii et al., 2012). This insertion phenomenon is only found in those tolerant genotypes originated from East Asia, due to evolutionary adaptable

abilities in acid soils (Fujii et al., 2012). Recently, a gene-specific marker, *HvMATE-21* indel (insert-depletion) was designed to the 3'-UTR of *HvMATE* gene (Bian et al., 2013). The polymorphism of this gene-specific marker has been verified in 50 genotypes differing in Al tolerance. All tested tolerant varieties and lines possessed the 21bp-deletion compared with sensitive ones. *HvMATE-21* has been mapped on chromosome 4H located between markers Bmag 353 and GBM1071. An association analysis has shown that the *HvMATE-21* indel could explain 66.9% of the variation for acid soil tolerance (Bian et al., 2013), demonstrating that it is more efficient than the two commonly-used SSR markers: Bmac310 and Bmag353 (Raman et al., 2003, Raman et al., 2002, Wang et al., 2007).

In contrast to *ALMT* genes (*TaMATE* genes) in wheat, *HvALMT1* gene (which is found located on chromosome 2H by analysis of wheat-barley addition lines) is not the major gene conferring to Al tolerance in barley (Gruber et al., 2011, Gruber et al., 2010, Ma et al., 2004, Wang et al., 2007). One possible explanation is that *HvALMT1* represents a related but different homologue of a lost *TaALMT1* gene previously present in the common ancestor of wheat and barley (Gruber et al., 2010). This gene can facilitate the secretion of malate and transport of other anions. Its primary expression localization is guard cells in stomata and root tissues, especially behind the root apex and at lateral root junctions (Gruber et al., 2011, Gruber et al., 2010). Although the over-expression of *HvALMT1* gene can greatly increase the efflux of malate and Al tolerance, the concentration of internal malate does not show significant changes as it can be replaced rapidly after secretion from root cells (Gruber et al., 2011). The Al tolerance relies primarily on Al-dependent malate efflux from root apices in wheat, which is attributed to an Al-activated anion transporter encoded by the ALMT1 gene (*TaALMT1*) located on chromosome 4DL (Sasaki et al., 2006). This finding was further proved by a strong correlation between the level of *TaALMT1* expression and Al tolerance in a number of wheat genotypes (Raman et al., 2008). *TaALMT1* can also result in Al-activated efflux of malate and enhanced Al tolerance when expressed in barley as well as other species, such as Xenopus oocytes and tobacco suspension cells (Delhaize et al., 2004, Pineros et al., 2008, Ryan et al., 2010, Sasaki et al., 2004).

2.2.3 Regulation of Al-responsive gene expression

Secretion of various organic anions including malate (wheat, *Arabidopsis*), citrate (barley, sorghum, maize, snapbean) or oxalate (buck wheat and taro) has been reported as the main external tolerance mechanism to deal with Al toxicity. The Al tolerance is highly correlated with organic acid biosynthesis (Houde and Diallo, 2008). So far, the Al tolerance mechanism in barley is limited as the citrate efflux are determined by constitutive expression of major genes (Delhaize et al., 2012). Transgenic studies have shown that the wheat Al-tolerance gene *TaALMT1* can increase Al-activated malate efflux and enhance the tolerance to Al toxicity in barley (Delhaize et al., 2004, Delhaize et al., 2009). Transgenic barley plants showed greater root growth, higher shoot biomass and 2-fold increase in grain production compared with untransformed controls when grown to maturity in acid soils (Delhaize et al., 2009). Improved ability to acquire the phosphorus from acid soils was also detected compared with control plants (Delhaize et al., 2009). These findings demonstrate that *TaALMT1* is effective in genetically engineering crop species for yield improvement in acid soils (Delhaize et al., 2009). Meanwhile, the transgenic wheat with the expression of the *SbMATE* tolerance gene isolated from sorghum showed increased Al-activated citrate efflux and higher Al^{3+}tolerance (Magalhaes et al., 2007). Although the genetic diversity in Al tolerance for wheat and barley largely relies on the constitutive expression of major genes, other genes which are induced by Al and essential in the secondary pathway of protection from Al toxicity are contributing to coordinated expression of Al tolerance genes (Delhaize et al., 2012).

In *Arabidopsis*, a zinc finger transcription factor STOP1 (sensitive to proton rhizotoxicity 1) (Iuchi et al., 2007) involved in tolerance to protons can regulate the expression of a large proportion of Al tolerance gene, such as *AtMATE*1, *AtALMT*1 and *ALS*3 (aluminium sensitive 3) (Delhaize et al., 2012, Liu et al., 2009, Sawaki et al., 2009). Although the activated STOP1 can up-regulate Al tolerance genes as well as other proton and Al-responsive genes (Sawaki et al., 2009), the genes conferring proton and Al tolerance generally were found to act independently (Delhaize et al., 2012). Similar to STOP1in *Arabidopsis,* ART1 (Al-resistant transcription factor 1) in rice also belongs to the C_2H_2-type zinc-finger transcription factors can regulate at least 31 Al tolerance genes. For example, the Al-activated MATE transporter gene named

OsFRDL4, a homolog of *HvAACT1* (Furukawa et al., 2007, Magalhaes et al., 2007), can be regulated by ART1 and greatly enhanced by short exposures to Al (Delhaize et al., 2012). The expression of other Al tolerance genes such as *STAR1-STAR2* complex, which facilitates UDP-glucose export, can be regulated by interaction between ART1 and its promoter region (Magalhaes, 2010, Yamaji et al., 2009). Meanwhile, a unique ART1-regulated gene *OsCDT3* found in rice, may contribute to high Al tolerance by binding Al and stopping Al import into root cells (Xia et al., 2013). In addition, because of high complementary pairing between miRNAs and their target genes, miRNAs may be one of molecular mediators associated with responses to Al toxicity (Lima et al., 2011). However, the involvement of miRNAs in Al toxicity is still largely unknown.

2.3 Salinity stress

2.3.1 Adverse effects of salinity stress

Salinity is a constraint that affects plant growth in a wide range of landscapes, such as estuarine marshes, lakes, and on irrigated and non-irrigated agricultural land (Jenkins et al., 2010). In crop production, salinity stress could reduce the number of plant tillers (monocotyledonous species) or dramatically restrict leaf and branch growth (dicotyledonous species). It has been proven that shoot growth could be more severely affected than root growth under salinity stresses (Barrett-Lennard and Shabala, 2013). The main adverse effects caused by salinity stress can be divided into two categories 1) osmotic effects and 2) ion (Na^+ and Cl^-) toxicity (Munns and Tester, 2008). The osmotic stress is caused by high concentrations of Na^+ and Cl^- that decreases soil water potential and pose immediate effects on plant growth by limiting availability of water to plants, whereas ion toxicity was due to the accumulation of Na^+ and/ or Cl^- in metabolically active tissues where these ions interfere with a large number of metabolic enzymes (Barrett-Lennard and Shabala, 2013, Flowers and Dalmond, 1992, Greenway and Munns, 1980).

2.3.1.1 Osmotic stress

The osmotic stress immediately reduces cell expansion in root tips and young leaves, and causes stomatal closure (Munns and Tester, 2008). For osmotic stress, abscisic acid (ABA) mediated accumulation of osmolytes and production of other compounds like glycine betaine can also be responsible (DeRose-Wilson and Gaut, 2011, Garthwaite et al., 2005, Ismail et al., 2009, Malik et al., 2009). Salinity affects stomatal conductance immediately, firstly and transiently owing to perturbed water relations and shortly afterward owing to the local synthesis of ABA (Fricke et al., 2004). Subsequently, the reduced rate of photosynthesis (caused by stomata closure) increases the formation of ROS, and increases the activity of enzymes that detoxify these species. ABA plays a central role in root-to-shoot and cellular signalling in osmotic stress as well as the regulation of growth and stomatal conductance (Davies et al., 2005, Zhu, 2002). However, studies of ABA deficient mutants revealed that a simple ABA control theory cannot explain the above plant performance under saline conditions. ABA can inhibit leaf elongation in barley by reducing the content of active *gibberellic acid* (GA) (Munns and Tester, 2008). Therefore, GA is regarded as another candidate for cellular signalling under the osmotic stress. In addition, accumulating evidence shows that the negative regulators of growth, the DELLA proteins, mediate the growth promoting effects of GAs in a number of species, and integrate signals from a range of hormones and abiotic stress conditions, including salinity (Achard et al., 2006) DELLA proteins may be the central coordinators that regulate plant growth to different environments (Munns and Tester, 2008). In many halophytes, proline or glycine betaine occurring at sufficiently high concentrations in leaves can contribute to the osmotic pressure (Flowers et al., 1977). In glycophytes, those compatible solutes generate a significant osmotic pressure and stabilize the tertiary structure of proteins to function as an osmolyte or osmoprotectants, although the concentrations of those solutes are not as high as those in halophytes (Rhodes et al., 2002). The role of glycine betaine accumulation functioning as an osmolyte has been suggested for in maize and 10%–20% higher biomass was observed under saline conditions with over-expression the glycine betaine accumulation gene *Bet1* (Saneoka et al., 1995).

2.3.1.2 Ion toxicity

For most species, Na^+ appears to be the major toxic ion as it could reach a toxic level earlier than Cl^- does (Munns and Tester, 2008). Therefore, the tolerance mechanism related with Na^+ exclusion and Na^+ transport under salinity stress has drawn great emphasis. Restriction of Na^+ uptake, compartmentation of Na^+ into vacuoles to maintain cellular homeostasis and active Na^+ exclusion back to the soil solution, control of xylem Na^+ loading and its retrieval from xylem are the main mechanisms for salt tolerance at the cellular level (Malik et al., 2009, Munns and Tester, 2008, Shabala and Cuin, 2008). Active transport of Na out of cytoplasm across the plasma membrane is mostly mediated by the Na^+/H^+ antiporters (Barrett-Lennard and Shabala, 2013, Maathuis, 2014, Shi et al., 2002). The salt overly sensitive (SOS1) gene has been suggested to function as a Na^+/H^+ antiporter located at the plasma membrane of plant cells, and to be involved in long distance Na^+ transport and in extrusion of Na^+ from the root meristem zone into the surrounding medium (Guo et al., 2009, Mian et al., 2011). SOS1 can affect the long-distance Na^+ transport by controlling Na^+ concentrations in xylem sap by unloading Na^+ from or loading Na^+ into xylem vessels depending on the severity of salinity stress (Guo et al., 2009). Transporters NHXs mediate both Na^+/H^+ and K^+/H^+ exchange and therefore affect both salinity tolerance and K^+ nutrition (Leidi et al., 2010, Venema et al., 2002). They can transport of either K^+ or Na^+ into the vacuole or endosome in exchange for H^+ efflux to the cytosol (NHX1-6) and Na^+ efflux out of the cell in exchange for H^+ influx into the cell (plasma membrane-bound NHX7-8) (Bassil and Blumwald, 2014). *NHX* overexpression lead to improved salt tolerance in diverse species (Apse et al., 1999, Liu et al., 2008, Ohta et al., 2002, Zhang et al., 2001) with a low Na^+/ K^+ cytosolic ratio by removing excess cytosolic Na^+ into the vacuole (Bassil and Blumwald, 2014), as well as attributing to K^+/H^+ exchange to regulate a vacuolar K^+ content (Barragan et al., 2012, Pilar Rodriguez-Rosales et al., 2008). The high-affinity K^+ transporters (HKTs) were first isolated from wheat (Schachtman and Schroeder, 1994). Sub-family1 of HKT transporters are only permeable to Na^+, while sub-family2 transporters are permeable to both K^+ and Na^+ (Corratge-Faillie et al., 2010, Rodriguez-Navarro and Rubio, 2006). HKTs can also play a role in Na^+ translocation to the shoots and Na^+ accumulation in leaves by contributing to Na^+ unloading from the ascending xylem sap and favouring Na^+ recirculation from leaves to roots (Mian et al.,

2011). The up-regulation of the HKT transport activity is highly correlated with a decrease in leaf Na^+ content (Davenport et al., 2007, Plett et al., 2010).

2.3.1.3 Disturbance of K^+ homeostasis

Recently, the third major constraint of salinity stress, namely its disturbance to cytosolic K^+ homeostasis was discovered (Shabala and Cuin, 2008). K^+ is an essential macronutrient that is required for diverse cellular processes such as osmotic regulation, maintenance of membrane potential, enzyme activity, protein and starch synthesis, respiration and photosynthesis (Schachtman and Schroeder, 1994, Ward and Schroeder, 1994). K^+ is also essential as a counter ion for the charge balance of ion transport across the plasma- and intra-organelle membranes. (Anschutz et al., 2014, Dreyer and Uozumi, 2011). High cytosolic K^+ levels are also essential to suppress activity of caspase-like proteases and endonucleases, thus reduce the cell risk in transition to PCD (Bortner et al., 1997). A strong positive correlation between the ability of plant roots to retain K^+ and salinity tolerance was revealed in a wide range of crop species in barley (Chen et al., 2007), wheat (Cuin et al., 2008) and lucerne (Smethurst et al., 2008). In barley, this K^+ retention ability conferred up to 60% of genetic variability in salinity stress tolerance (Anschutz et al., 2014, Cuin et al., 2012). Since excessive Na^+ ions repress various important cellular processes many of which are directly correlated with K^+ transport and essential functions of K^+, it is well recognized that K^+ alleviates toxic effects of Na^+, and that a high K^+/ Na^+ ratio in shoots, especially in leaves, is important in glycophytes for enhanced salinity tolerance (Hauser and Horie, 2010). It was also shown that supply of K^+ fertilizers has a beneficial impact on plant performance under salinity stress (Siringam et al., 2013, Umar et al., 2011). Massive K^+ leak from cytosol has been observed under saline conditions. It is suggested that outward-rectifying depolarization-activated K^+ channels (GORK in *Arabidopsis*) are the main pathway for the salinity-induced K^+ efflux from the cytosol (Shabala and Cuin, 2008). An important factor contributing to massive K^+ leak is ROS. In salt-stressed plants ROS levels are known to be much higher (Jacoby et al., 2011). Several types of K^+-permeable channels, including non-selective ion channels (NSCC) (Demidchik et al., 2003) and GORK (Demidchik et al., 2010) are activated by ROS, providing an additional (independent of membrane depolarization) pathway for K^+ leakage from the cytosol and even the vacuoles (Demidchik, 2014). If the K^+ leakage process takes too long, the vacuolar K^+ pool is

depleted and the cell collapses. Based on above observations the optimal Na^+/K^+ ratio can be maintained by either restricting the Na accumulation or preventing the K^+ loss from the cell (Shabala and Cuin, 2008), and Na^+/K^+ ratio has been regarded as an efficient indicative index for salinity tolerance.

2.3.1.4 Detrimental effects of ROS

A plant's ability to detoxify ROS and/or repair ROS-induced damage to key cellular structures has also been considered as a key feature of salinity tolerance especially in halophytes, which are adapted to high saline soils (Maksimovic et al., 2013). A comparison of photosynthetic response between the halophyte *Thellungiella salsuginea* and the glycophyte *Arabidopsis thalina* during salt stress revealed that: electron transport through PSII and the activity of plastid terminal oxidase (PTOX) protein (diverting up to 30% of total PSII electron flow to O_2) is substantially increased whereas they are inhibited in *Arabidopsis* (Stepien and Johnson, 2009), suggesting that alterative electron sinks have the potential to decrease salt-induced ROS production in halophytes (Bose et al., 2014). Moreover, ROS production is critically dependent on K^+ availability. Increases in the severity of K^+ deficiency were also associated with enhanced activity of enzymes involved in detoxification of H_2O_2 and utilization of H_2O_2 in oxidative processes, and K^+ deficiency also caused an increase in NADPH-dependent $O_2^{\bullet -}$ generation in root cells (Cakmak, 2005). Thus, increasing the K^+ nutritional supply can reduce the detrimental build-up of ROS either by enhancing photosynthetic electron transport or by inhibiting the membrane-bound NADPH oxidases and lead to enhanced salinity tolerance (Shabala and Pottosin, 2014).

2.3.2 Genes and QTL identified for salinity tolerance

Apart from the Na^+ exclusion genes *NHX*, *SOS* and *HKT* discussed in section 2.3.1.2, two major genes for Na^+ exclusion named *Nax1* and *Nax2* have been identified in a wheat line from a cross between *Triticum monococcum* and a durum wheat cultivar (James et al., 2011). Both *Nax* genes appeared to originate from the wheat relative, *Triticum monococcum*. *Nax1* was located on chromosome 2A (Lindsay et al., 2004) and was designated as an Na^+ transporter of the *HKT* gene family *HKT7* (*HKT1;4*) (Huang et al., 2006). *Nax2* was located on chromosome 5A and identified as *HKT8* (*HKT1;5*)

(Byrt et al., 2007). The *Nax* genes are not present in hexaploid wheat (Huang et al., 2008). It has been shown that by introgressing *Nax* genes from *T.monococcum* into hexaploid wheat, the leaf Na^+ concentration is reduced and the proportion of Na^+ stored in leaf sheaths is increased. Thus, these extra Na^+-excluding genes have the potential to increase the salt tolerance of bread wheat (Colmer et al., 2005). In durum wheat, these genes enhanced removal of Na^+ from the xylem, resulting in low Na^+ concentrations in leaves (James et al., 2006). *Nax1* removes Na^+ from the xylem in roots and the lower parts of leaves including the leaf sheaths, while *Nax2* removes Na^+ from the xylem only in the roots (James et al., 2006). *Nax1* can induce a high sheath:blade ratio of Na^+ concentration, and this unique function of *Nax1* would help enhance the tolerance to salinity stress in waterlogged soils, especially during the plant vegetative phase. The *Nax2* locus confers a reduced rate of Na^+ transport from roots to shoots by delivering Na^+ from the root xylem (Davenport et al., 2005, James et al., 2006). Proteins encoded by group 1 *HKT* genes of *AtHKT1;1* from *Arabidopsis* and *OsHKT1;5* from rice, and, reduce transport of Na^+ to shoots (Moller et al., 2009, Sunarpi et al., 2005). An *OsHKT1;5*-like gene was considered a possible *Nax2* candidate12 because *OsHKT1;5* induces a similar reduced rate of Na^+ transport from roots to shoots and increases plant salinity tolerance in rice (Munns et al., 2012, Ren et al., 2005).

Among all cereal crops, barley is arguably one of the most tolerant to salinity stress and can tolerate high concentration of Na^+ in leaf blades (Munns and Tester, 2008). Yet, salinity stress can still restrict barley biomass production and yield. It is imperative to enhance salt tolerance of barley through genetic improvement, especially when the diversity of allele variations of modern cultivated barley is reduced during domestication (Qiu et al., 2011, Russell et al., 2004). Salinity tolerance in barley is a complex quantitative trait controlled by multiple major or minor genes (Mano and Takeda, 1997, Qiu et al., 2011, Xue et al., 2009). There have been many studies showing that QTL for salt tolerance can be associated with numerous traits (Qiu et al., 2011). For example, genetic diversity analysis with 48 barley accessions found associations between agronomic traits including plant yield and salinity stress (Eleuch et al., 2008). Using different segregating mapping populations, QTL for salinity tolerance at germination or seedling stages were identified by evaluating leaf chlorosis, plant survival, the tiller number, plant height, spike number, dry weight , grain yield, shoot Na^+ and K^+ contents, Na^+/K^+ ratio (Xue et al., 2009). Using a DH population derived

from YYXT (tolerant) and Franklin (sensitive), Zhou et al. (2012) identified five significant QTL for salinity tolerance located on chromosomes 1H, 2H, 5H, 6H and 7H, respectively. Those loci can explain more than 50% of the phenotypic variation (Zhou et al., 2012). More recently, Shavrukov et al. (2010) found a single QTL for salinity tolerance, named *Nax3*, which located on the short arm of chromosome 7H and could reduce the shoot sodium content of plants grown in 150 mM NaCl by 10-25%. Rivandi et al. (2011) identified a QTL for salt exclusion in barley on the long arm of chromosome1H, called *HvNax4*. This locus co-segregated with a barley homologue of the *SOS3* gene which is a salinity tolerance gene in *Arabidopsis*. The *HKT* families involved in Na^+-K^+ homeostasis are considered to play an important role in salinity tolerance and *HvHKT1* which mediates Na^+ uniport in roots was cloned (Haro et al., 2005).

2.4 The interaction between waterlogging and other stresses

2.4.1 Waterlogging stress and Al toxicity

Al toxicity mainly occurs in the humid northern temperate zone and the humid tropics (Zeng et al., 2013). Soil acidification can be closely associated with soil waterlogging in high rainfall regions (Khabaz-Saberi and Rengel, 2010). The duration of waterlogging can be either short-term or long-term, depending on the amount of rain, evapotranspiration and soil structure (Malik et al., 2002). Even short-term/ transient soil waterlogging (within a few hours or days) can have considerable effects on root growth /survival, nutrient uptake and yield (Barrett-Lennard and Shabala, 2013, Malik et al., 2002). Few studies have been focused on the interaction between the waterlogging stress and Al toxicity. In respect of the ion toxicity, it was found that waterlogging could increase concentrations of Al in acidic soils, which was linked with an increase in shoot concentrations of Al in wheat genotypes (Khabaz-Saberi and Rengel, 2010). Tolerance to Al toxicity has also been shown to improve grain yield in transiently waterlogged soils in some wheat genotypes (Khabaz-Saberi et al., 2014). However, no studies for the plausible mechanism underlying the effects of short-term waterlogging (hypoxia) on Al tolerance were conducted yet.

Both waterlogging and low-pH/Al stresses were found to affect the root plasma membrane potential (MP) balance. While the low-pH alone could depolarize the MP through rapid H^+ influx into the root tissue (Bose et al., 2010a), there is still controversy whether Al induces depolarization or hyper-polarizition (Bose et al., 2010b). In contrast, oxygen deprivation was able to rapidly depolarize the membrane potential (Teakle et al., 2013, Zeng et al., 2014). It has to be mentioned that the functional plasma membrane H^+-ATPase is a key element in maintaining MP, which could also regulate internal pH and membrane energization for the secondary H^+-coupled transport (Duby and Boutry, 2009). As most membrane transporters located on plasma and organelle membranes are voltage-gated and K^+ is the most abundant cation in the cytosol, disturbance of the intracellular K^+ homeostasis is inevitable (Ward et al., 2009, Zeng et al., 2014). Some studies found Al toxicity could induce K^+ efflux or reduces K^+ uptake to balance the charge created by the exudation of organic acid (Ma et al., 2001, Osawa and Matsumoto, 2002), while some studies have reported an Al-induced decrease in net K^+ efflux (Bose et al., 2011, Bose et al., 2010b, Olivetti et al., 1995, Sasaki et al., 1995, Tanoi et al., 2005). Similarly, significant K^+ leak in wheat or barley roots (Buwalda et al., 1988, Zeng et al., 2014) and K^+ uptake in one waterlogging-tolerant haplotype grass was observed under hypoxia stress (Teakle et al., 2013, Zeng et al., 2014). The different root tissues (the elongation zone and mature zone) or genotypes for assessment should be taken into consideration when evaluating the effect of hypoxia stress (Pang et al., 2006). Despite controversy around experimental results of K^+ kinetics, it is believed that higher ability to retain K^+ in the plants' cells often is closely linked with the increased stress tolerance to salinity, heavy metals, heat, oxidative stress and waterlogging stress (Shabala et al., 2011).

While the generation of ROS during abiotic stresses could potentially cause oxidative damage to cells, ROS could function as signal transduction molecules mediating plant responses to pathogen infection, environment Al toxicity, programmed cell death and different developmental stimuli (Mittler et al., 2004, Torres and Dangl, 2005). Accumulation of H_2O_2 in the apoplast is thought to be involved in acclimation responses of plants to drought and salt stresses, including growth and cell wall strengthening (Miller et al., 2010). Hypoxia stress and the re-entry of oxygen following drainage can induce the ROS accumulation in plants roots (Biemelt et al., 2000, Xu et al., 2013). ROS produced by the membrane NADPH oxidase are involved in signalling through a ROP

rheostat and could mediate waterlogging-induced ethylene-controlled adaptation (Banti et al., 2013, Kreuzwieser and Rennenberg, 2014, Shabala et al., 2014, Steffens et al., 2013). Internal ROS production can be triggered by Al, accompanied by the respiration inhibition and ATP depletion, the phenomenon of which is well consistent with the inhibition of cell growth and the inhibition of root elongation (Yamamoto et al., 2002). The role of ROS in stress signalling provides a clue for studying the interaction between waterlogging stress and Al toxicity.

The decrease in redox potential induced by flooding increases the accumulation and solubility of many toxic metals, including manganese (Jackson and Drew, 1984). High concentrations of Al and Mn can concur in soils, restricting crop production in acid and high-Mn soils. Heavy rainfall can increase the concentrations of available Al and Mn (Yang et al., 2009). Although high concentrations of Mn and Al can concur in acid soil, the tolerances of plants to Al and Mn do not necessarily coincide (Nelson, 1983). This raises questions as to how plants adapt to both Mn and Al toxicity and how Mn interacts with Al in plants. Previous reports have indicated that Al can reduce Mn accumulation in barley, corn (*Zea mays*) (Clark, 1977), wheat (Blair and Taylor, 1997), cowpea (*Vigna unguiculata*) (Taylor et al., 1998) and soybean (*Glycine max*) (Yang et al., 2009). One explanation for this alleviation is that Al may have an antagonistic effect on Mn uptake by plant roots. However, the exact mechanism is still poorly understood. The results of Kopittke et al. (2011) revealed that the effects of cations on Mn nutrition were related to the electrical potential (wo0) of the root cell membrane surface, which provided new clues about the interaction between Al and Mn (Wang et al., 2015).

2.4.2 Waterlogging and salinity stresses

Waterlogging often occurs in saline soils due to intensive irrigation in agricultural production systems, rise of saline water tables, and seawater intrusion in coastal environments (Carter et al., 2006, Zeng et al., 2013). The general area of waterlogging and salinity interactions was first discovered ~30 years ago, mainly by researchers interested in understanding some of the constraints to irrigated agriculture (Barrett-Lennard and Shabala, 2013). It is suggested that a salt-tolerant genotype would also have a higher tolerance of the combination of waterlogging and salinity stress than a salt-sensitive one, based on the observation of increased antioxidant activity in salinity-

tolerant genotypes under waterlogging and salinity stresses (Turkan et al., 2013). Differences in salt tolerance of three poplar genotypes were found dependent on their ability to restrict salt transport from roots to leaves (Chen et al., 2002), and more tolerant poplar individuals had consistently lower shoot Na^+ contents and Na^+: K^+ ratios than those less tolerant ones (Fung et al., 1998). It was demonstrated that over-accumulation of Na^+ and Cl^- in shoots under waterlogged conditions is greater than for salinity alone since energy (ATP) crisis caused by waterlogging-induced anoxic stress dampened the exclusion of Na^+ and Cl^- (Teakle et al., 2010). When salinity and root hypoxia concur, it could result in a more severe reduction in the uptake of K^+ and increased shoot Na^+ and Cl^- concentrations in non-halophytes (Barrett-Lennard, 2003), although this usually does not occur in wetland halophytes (Colmer and Flowers, 2008, English and Colmer, 2011, Malik et al., 2009). Combined salinity and waterlogging stress caused more severe damages than salinity stress by comparison of damage index in different growth stages and leaf Na^+/K^+ ratio (Reguera et al., 2015). Disturbance of K^+/Na^+ homeostasis has been defined as central for the severe effects of combined salinity and waterlogging stress (Zeng et al., 2013). The reduced activity of plasma membrane SOS1Na^+/H^+ antiporters that actively expel Na^+ from the cytosol can play an important role in the elevated Na/K ratio under salinity-waterlogging combined stress, due to the energy deficits and reduced activity H^+-ATPase in poorly aerated roots (Barrett-Lennard, 2003, English and Colmer, 2011). Anoxic stress causes an energy crisis, which will supress transport processes associated with 'exclusion' of Na^+ and the activity of H^+-ATPase that maintains H^+ gradients across membranes and motivate the active secondary transport of Na^+ (Barrett-Lennard, 2003, Greenway and Munns, 1980, Munns, 2005). Potential sites of Na^+ transport in roots that rely on trans-membrane H^+ gradients, and that might therefore be impaired by O_2 deficiency, include (1) retrieval from the xylem, possibly via high-affinity Na^+ transporters (e.g. HKT) (Byrt et al., 2007, Huang et al., 2006, James et al., 2006), Na^+/H^+ antiporters (e.g. SOS1) (Shi et al., 2002) or cation/H^+ exchangers (CHX) (Hall et al., 2006); (2) accumulation of Na^+ in root vacuoles by tonoplast Na^+/H^+ antiporters (e.g. NHX1) (Pardo et al., 2006, Xue et al., 2004) and (3) efflux of Na^+ via plasma membrane Na^+/H^+ antiporters (e.g. SOS1) (Martinez-Atienza et al., 2007, Oh et al., 2007) . It has been found that tolerance to combined salinity and root-zone O_2 deficiency in *L. tenuis* was associated with lower shoot concentrations of Na^+ and Cl^- because of reduced net loading in the xylem, and higher root porosity from constitutive aerenchyma formation (Teakle et al., 2010, Teakle et al., 2007).

Furthermore, energy deficits in poorly aerated roots can also lead to reduced uptake of K^+, which is closely related with upregulated depolarization-activated outward-rectifying (GORK in *Arabidopsis*) channels and repressed K^+ uptake associated inward-rectifying (KIR) K^+ channels (Barrett-Lennard and Shabala, 2013, English and Colmer, 2011, Shabala and Cuin, 2008, Zeng et al., 2013).

Under salinity stress, high concentrations of Na^+ cause growth retardation and may result in plant death because of drastic changes in ion and ROS homeostasis (Li et al., 2014). In addition, salinity stress induces production of ethylene, which can function as a downstream signal and alter gene expression (Wang et al., 2002). Enhanced synthesis of ACC, the natural precursor of ethylene can reduce salinity tolerance in rice (Dong et al., 2011, Steffens, 2014). Moreover, during salinity stress NADPH oxidases and PA oxidases are activated which elevates ROS concentration (Abogadallah, 2010). Excessive ROS production switches on the PCD program and may significantly supress the plant growth. Similarly, ethylene and ROS are integrated to induce aerenchyma development for waterlogging tolerance. For example, exogenous application of H_2O_2 stimulates lysigenous aerenchyma emergence in rice stems (Steffens et al., 2011). Moreover, one of the highly upregulated genes during waterlogging in maize is *Rboh*, is a major ROS producer (Rajhi et al., 2011). In addition, the accumulation of metal ions and mitochondrial dysfunction during waterlogging stress also increase ROS levels (Shabala et al., 2014). Applying NADPH oxidase inhibitor diphenyleneio donium chloride (DPI) alone prevents the aerenchyma formation (Parlanti et al., 2011). These findings support that ROS are essential for aerenchyma development and transduction of an ethylene signal to initiate the waterlogging tolerance (Petrov et al., 2015).

The study of non-symbiotic hemoglobins (nsHbs) in *Arabidopsis* showed that class I nsHbs which are immediately induced under oxygen-deficiency stress are involved in other stress responses (Hunt et al., 2002, Trevaskis et al., 1997). More recently, it was shown by studying the expression pattern of nsHb genes from maize (*ZmHb*) in transgenic tobacco plants that *ZmHb* was involved in responses to submergence stress and high-salt and osmotic stresses (Zhao et al., 2008).

2.5 Effects of flowering time on plant stress tolerance

Flowering, a transition from vegetative to reproductive phase, is the most dramatic change in the plant's life cycle (Seo et al., 2009). To maximize reproductive success, plants have evolved a complicated mechanism determining flowering time in response to both environmental factors, and endogenous signals (Baurle and Dean, 2006). It is also known that plant flowering time is regulated by various abiotic stresses, such as nutrient deficiency, heat and cold (Balasubramanian et al., 2006, Baurle and Dean, 2006, Kim et al., 2004). Thus understanding the interaction between flowering time and environmental stressors will provide significant clues for mechanisms of plant stress tolerance.

As an important trait for plant development, flowering time is determined by external environmental cues (photoperiod and vernalisation) and endogenous developmental signals (Yang et al., 2014). The major genes that control flowering time in barley in response to environmental cues are *VRNH1*, *VRNH2*, *VRNH3*, *PPDH1* and *PPDH2* (candidate gene *HvFT3*) (Casao et al., 2011). *VRNH1* (located on chromosome 5H) acts as a promoter of flowering, is induced by vernalisation, and regulates the transition to the reproductive stage at the shoot apex (Hemming et al., 2008). *VRNH2* is a floral repressor that delays flowering until plants are vernalized (Yan et al., 2004). *HvFT1*, candidate gene for *VRNH3*, is a homolog of the *FLOWERING LOCUS T* gene (*FT*) of *Arabidopsis thaliana* (Faure et al., 2007, Yan et al., 2006). *PPDH1* (located on chromosome 2H) confers sensitivity to a long photoperiod and accelerates flowering under long days (Turner et al., 2005). In particular, the function of *PPDH2* is assumed to be limited to the ability of a short photoperiod to promote flowering (Casao et al., 2011). Most of barley's natural variation in photoperiod response is associated with allelic differences in the photoperiod genes *PPD-H1* and *PPD-H2* (Hemming et al., 2008). Flowering in plants such as wheat, barley, pea (*Pisum sativum*) and *Arabidopsis thaliana* is strongly promoted under long-day (LD) conditions, with transcriptional activation of *FLOWERING LOCUS T*-like genes (*FT1* in barley) being a key determinant of the flowering response (Boden et al., 2014).

More importantly, flowering time has been found to be associated with plant stress tolerance (Yaish et al., 2011). In perspective of the interaction between flowering time

and stress tolerance, it was demonstrated that in winter wheat and barley genotypes with long exposures to non-freezing cold temperatures accelerate flowering time (vernalisation) and improve freezing tolerance (cold acclimation) (Dhillon et al., 2010). The close link between flowering time and stress tolerance has been illustrated by studies in the following three different subjects. Firstly, the interplay between environment stresses and flowering has been illustrated by the studies of plant transcription factors (TFs). These TFs can variously function as transcriptional activators or repressors and govern downstream gene expression either cooperatively or independently in stress signalling (Hirayama and Shinozaki, 2010, Yang et al., 2014). For example, *BBX24*, a zinc finger transcription factor gene, from *Chrysanthemum morifolium* was found to be associated with both flowering time and stress tolerance (Hirayama and Shinozaki, 2010). NAC proteins as plant specific TFs have been found to play roles in formation of embryos and flowers in petunia (Souer et al., 1996) and also in senescence of *Arabidopsis* (Balazadeh et al., 2010). AtRD26 /ANAC072 is induced by dehydration, high salinity and exogenous ABA, and it is thought to be involved in a novel ABA-mediated stress-signalling pathway in *Arabidopsis* (Fujita et al., 2011). Over-expression of *BnNAC485* enhanced tolerance to abiotic stress in both *Brassica napus* and *Arabidopsis thaliana* (Ying et al., 2014). Secondly, a hormonal involvement has also been reported. A cross-regulatory mechanism implicating ABA, salicylic acid, jasmonates and auxin in the floral initiation process was reported in soybean (Avanci et al., 2010, Diallo et al., 2014, Wada et al., 2010). For example, methyl jasmonate (MeJA) is a volatile compound initially identified from flowers of *Jasminum grandiflorum* and found to be distributed ubiquitously throughout the plant kingdom where it acts as a vital cellular regulator that mediates diverse developmental processes and defence responses against biotic and abiotic stresses in maize, *Pharbitis nil* and *Chenopodium rubrum* (Albrechtova and Ullmann, 1994, Cheong and Choi, 2003, Maciejewska et al., 2004). A recent report showed that jasmonate caused a flowering delay and enhanced protection against biotic stress in *Arabidopsis* (Chehab et al., 2012). Thirdly, epigenetic changes associated with plant development can also affect stress tolerance (Yaish et al., 2011). For example, loss of DNA methylation can reduces the ability of *Arabidopsis* plants to tolerate salt stress conditions by reducing the expression of the sodium transporter gene (*AtHKT1*) (Baek et al., 2011). Correspondingly, the reduction in cytosine methylation by treating plants with the cytosine methyltransferase inhibitor 5-azacytidine promotes flowering in the vernalisation-requiring *Arabidopsis*

and in wheat (Yaish et al., 2011). In *Arabidopsis*, KINASE BINDING PROTEIN1 (SKB1) binds to chromatin and increases the histone 4 Arg3 (H4R3) symmetric dimethylation (H4R3sme2) level, which in turn leads to the down-regulation of FLC expression as well as a number of stress-responsive genes. As a result, the phenotypes present in this mutant exhibit salt hypersensitivity, late flowering, and growth retardation (Zhang et al., 2011). Moreover, plants can also adapt their growth and developmental processes in response to environmental conditions. For example, under stress conditions such as drought, high salt, high temperature, and high light intensity, physiological processes are induced to reduce the cellular damage caused by stresses and, at the same time, alter developmental timing to complete their life cycle in a timely manner (Yaish et al., 2011).

2.6 Summary

In this literature review, the recent progress on studies of three categories of stresses has been discussed. In the section of 'waterlogging stress', the adverse effects of waterlogging, the tolerance mechanisms on both physiological/genetic aspects and the breeding perspectives in waterlogging tolerance were summarized. In the section of 'Al toxicity', the deleterious effects of Al on plant metabolism, (including oxidative stress, disturbance of Ca homeostasis and effects on plant nutrition) and the genetic mechanisms of Al tolerance were discussed. In the section of 'salinity stress', the adverse effects of salinity stress featured with ion toxicity and the genes/QTL conferring salinity tolerance were reviewed. The effects of combined waterlogging/salinity stress and combined waterlogging stress/ Al toxicity were also discussed. In addition, evaluation of stress tolerance was mostly conducted at the plant vegetative stage and the switch to reproductive stage can modify the plant sensitivity to stresses. Therefore, the interplay between flowering time and stress tolerance was summarized.

Realising the gaps of previous studies, experimental evidences will be provided in this study to address the following research aspects in barley: 1) identification of the genetic loci conferring salinity tolerance under waterlogged and drained soils. 2) the possible mechanisms underlying plant tolerance to combined waterlogging stress and Al toxicity. 3) allelic variations of the major Al tolerance gene *HvAACT1*.

Chapter 3 Quantitative trait loci for salinity tolerance under waterlogged and drained conditions and their association with flowering time[2]

3.1 Introduction

Salinity stress is one of the major abiotic stresses affecting crop production and many saline soils are also prone to waterlogging, with the resulting root hypoxia reducing growth of dryland cereals (Setter and Waters, 2003, Xu et al., 2012). The main adverse effects caused by salinity stress can be divided into two major categories 1) osmotic effects and 2) ion toxicity (Na^+ and Cl^-) (Munns and Tester, 2008). The osmotic stress can pose immediate effects on plant growth by limiting availability of water to plants (Barrett-Lennard and Shabala, 2013). Specific ion toxicity in the shoot takes a longer time to impact plant growth (days or weeks), and shows less effect than the osmotic stress, especially at low to moderate salinity levels (Barrett-Lennard and Shabala, 2013, Munns and Tester, 2008), although in roots NaCl-specific programmed cell death is observed within hours afters salinity stress onset (Shabala, 2009). Yet, the main site of Na^+ toxicity for most plants is the leaf blade, where Na^+ accumulates, after being deposited in the transpiration stream, rather than in the roots (Munns, 2002, Munns and Tester, 2008). The cytosolic K^+/Na^+ ratio has been repeatedly named as a key determinant of plant salt tolerance (Anschutz et al., 2014, Barrett-Lennard and Shabala, 2013, Colmer et al., 2006, Cuin et al., 2003, Shabala and Cuin, 2008). The optimal cytosolic K^+/Na^+ ratio can be maintained by either restricting Na^+ accumulation in plant tissues or by preventing K^+ loss from the cell (Shabala and Cuin, 2008).

Barley has a relatively highly salt-tolerance among cereal crops and can tolerate a high concentration of Na^+ in leaf blades (Munns and Tester, 2008). Salinity tolerance in barley is a complex trait controlled by multiple genes (Qiu et al., 2011). There have been many reports on QTL related with salt tolerance which has been evaluated by

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numerous traits at the whole plant level. These include yield and agronomic traits, leaf chlorosis, plant survival, shoot sodium content and Na/K ratio (NAK) (Eleuch et al., 2008, Qiu et al., 2011, Xue et al., 2010, Zhou et al., 2012). A single major QTL for salinity tolerance was identified from a Chinese landrace. This QTL was located on chromosome 2H explaining nearly 50% of the phenotypic variation (Xu et al., 2012). *HvNax3* and *HvNax4*, conferring a mechanism of sodium exclusion, were found on the short arm of chromosome 7H and the long arm of chromosome 1H, respectively (Rivandi et al., 2011, Shavrukov et al., 2010). *HvNax4* has been proved as a barley homologue of the *SOS3* salinity tolerance gene of *Arabidopsis* (Rivandi et al., 2011). The *HvHKT1* gene conferring Na^+ uniport in barley roots has been cloned (Qiu et al., 2011).

Excess water in the root zone of land plants is detrimental or lethal when it forms a barrier between soil and air free transfer of gases, such as O_2 and CO_2 (Drew, 1997), with the effect of inadequate oxygen supply being most significant. In addition to the elemental toxicities to the sensitive root tips caused by O_2 deficiency, increased concentration of secondary metabolites such as phenolics and volatiles fatty acid could become injurious in the rhizosphere (Pang et al., 2007, Shabala, 2011). Waterlogging stress is often comorbid with salinity. Over-accumulation of Na^+ and Cl^- in shoots under waterlogged conditions is greater than for salinity alone since energy (ATP) stress caused by waterlogging-induced anoxic stress dampened the exclusion of Na^+ and Cl^- (Teakle et al., 2010). The active transport of Na^+ out of the plant mediated by plasma membrane Na^+/H^+ antiporters (Qiu et al., 2003, Shi et al., 2000) is suppressed when activity of the plasma membrane H^+-ATPase is reduced under an anoxia environment (Barrett-Lennard and Shabala, 2013). When salinity and root hypoxia occur together, K^+ export channels may be activated by ROS produced under stress conditions (Demidchik et al., 2003, Demidchik et al., 2007, English and Colmer, 2011), leading to a more severe reduction in the uptake of K^+ (Malik et al., 2009) as well greatly increased leakage of K^+ from plant roots.

Flowering time in plants (or heading date in crops), an important trait for plant development, has also been reported to be associated with stress tolerance (Gao et al., 2014, Wada et al., 2010, Yaish et al., 2011). The interplay between environmental stressors and flowering time has been investigated by plant transcription factors (TFs)

at the genetic or epigenetic level and through hormonal interaction (Chehab et al., 2012, Yaish et al., 2011, Ying et al., 2014). However, no direct evidence from the perspective of QTL mapping or gene exploration has been provided yet.

In this study, 175 double haploid (DH) lines from a cross between YSM1 and Gairdner were genotyped with Diversity Arrays Technology (DArT), which ended up with more than 5,800 single nucleotide polymorphism (SNP) and 13,500 DArT markers. About 8,500 markers with low distortion level and no missing data were selected to construct a genetic map. Salinity tolerance was screened under various environments, i.e. summer trial and winter trial, and well-drained or waterlogged saline potting mixture. QTL were mapped for salinity tolerance based on plant survival as described in previous studies (Xu et al., 2012, Zhou et al., 2012).

3.2 Materials and Methods

3.2.1 Plant materials

A double haploid (DH) population consisting of 175 lines, derived from a cross between Yangsimai 1 (YSM1) and Gairdner was used to identify QTL conferring salinity tolerance. YSM1 is originated from China with medium tolerance to both salinity (Wu et al., 2013) and waterlogging stresses (unpublished data). Gairdner is an Australian variety and highly-sensitive to salt stress (Chen et al., 2008). However, Gairdner also showed medium tolerance to waterlogging stress, compared with two other well-known waterlogging-intolerant varieties Franklin and Naso Nijo (Pang et al., 2004). In addition, the four barley genotypes: Baudin, Franklin, TAM407227 and Naso Nijo, were included as controls.

3.2.2 Evaluation of salinity tolerance under drained and waterlogged environments

Both summer and winter trials for salinity tolerance screening were conducted in glasshouses located at Mt Pleasant Laboratories, Launceston, Tasmania. Two treatments were used: 200 mM NaCl with drained condition (SalinityD) as described

before (Zhou et al., 2012) and waterlogged (SalinityW) with 200 mM NaCl solution. In the summer trials (from February to April in Year 2014), two replicates were included for each treatment. Eight 40-L containers (each including 18 lines) filled with commercial potting mixture were used for each replicate. All replicates were arranged in a randomized block design. Glasshouse settings for plant growth were 25/15 (±5) °C for day/night temperature under natural daylight. The SalinityD and SalinityW treatments were started at the three-leaf stage, and lasted four weeks. The drainage system and application system connected between containers and pumps for salinity treatment were as previously described (Zhou et al., 2012). The system approached a steady state where, after 4-5 watering cycles, NaCl additions were minimal and only water was added to replace evaporation and transpiration (Zhou et al., 2012). For SalinityW treatment the saline solution (200 mM NaCl) was kept in the containers rather than being drained (just above the surface). Winter trials were conducted between June and August in Year 2014. Glasshouse settings for plant growth were 15 /10 (±5) °C for day/night temperature under natural daylight. All treatments were the same as summer trials. The control experiment was not conducted since it has been proved that different varieties or DH lines grown in the same potting mixture but with no salt added showed no apparent symptoms of leaf chlorosis or wilting (Xu et al., 2012, Zhou et al., 2012). Salinity tolerance was assessed by combining scores for leaf chlorosis and plant survival two weeks after SalinityW treatment for summer trials and three weeks after SalinityW treatment for winter trials, four weeks after SalinityD treatment for summer trial and five weeks after SalinityD treatment for winter trial (0 = no damage and 10 = all dead; scores between 0–5 are basically the level of leaf chlorosis and the number of dead leaves and score 6–10 are the percentage of plant survival as well as dead leaves and leaf chlorosis of survived plants).

3.2.3 Assessment of plant flowering time

Plant development stages (flowering time) was scored only in summer trials as the effect of vernalisation cannot be clearly detected in winter time. It was assessed after 3-week salinity and SalinityW treatments with score ‘1’ = staying at tillering stage, ‘2’ = stem extension and booting stage and ‘3’ = heading stage (Zadoks et al., 1974).

3.2.4 Leaf Na^+ and K^+ contents measurement

In summer trials, after three-week SalinityD and SalinityW treatments, the youngest fully expanded leaf was collected and immediately stored in a 1.5ml microcentrifuge tubes at -20°C. The sap from leaves was extracted by the freeze-thaw method (Cuin et al., 2008). After centrifugation at 10,000g for 3min, the extracted sap sample was diluted 50 times with double distilled water and analysed for Na and K contents using a flame photometer (PF97, VWR International, Murarrie, Australia). Na/K ratio was then calculated. Four replicates of measurement were taken for every leaf sample (Zeng et al., 2013).

3.2.5 Marker development using comparative genomics

The high level of conserved genomic synteny between the grass species *Brachypodium* and barley provides useful clues in marker development, fine mapping and gene cloning. The sequences of *Brachypodium* showing collinearity with specific barley chromosome region were downloaded from Phytozome Database (Version 10.3). Those sequences were BLAST against the barley genome using IPK blast server (http://webblast.ipk-gatersleben.de/barley/), with E-value threshold of 10^{-5}. The best hit sequence was selected for primer design to amplify a band of 800~1000bp length. The PCR products were then sequenced and aligned with sequence analysis software DNAMAN (version 7.0; Lynnon Biosoft, USA). SNPs between the parents of a given population were exploited to develop cleaved amplified polymorphic sequence (CAPS) marker using dCAPS Finder 2.0 (Neff et al., 2002). PCR products were digested with appropriate enzymes from New England Biolabs (NEB) based on target sequences differences and separated on 2% agarose gels.

Cloning methods were used to purify PCR products for sequencing. After performing PCR to amplify the gene of interest with high fidelity DNA polymerase, PCR product was checked by running agarose gel (1%~2%) electrophoresis. The desired band was cut and purified using the QIAquick® Gel Purification Kit (Qiagen). A total of 3 µL purified PCR product was mixed with the pGEM®-T Vector System I: 1 µL pGEM®-T Vector, 5 µL ligation buffer and 1 µLT4 DNA Ligase reaction buffer and incubated

overnight at 4°C. The 10 µL cloning reaction mixture was transformed into *Escherichia coli* JM109 cells using heating shock at 42°C for 90 secs which was followed by 2 min-incubation on the ice. The cells were transferred to 1mL LB liquid medium for 1h-shaking-culture at 37°C and then spread on the LB plate containing 100 µg/mL ampicillin, 40 µL 100mM IPTG (Isopropyl β-D-1-thiogalatopyranoside) and 40 µL 20 mg/Ml X-Gal (5-Bromo-4-chloro-Indoly- β-D-Galactoside). The plates were placed at 37°C overnight (about 16h). Approximately 8-10 white (positive) colonies (with recombinant plasmids carrying PCR fragments of interest) were selected and cultured by shaking overnight at 37°C in 8~10 mL LB liquid media containing 100 µg/mL ampicillin. The plasmid DNA was isolated with a the Miniprep kit (Qiagen). After measuring the DNA concentration with a Nanodrop ND-1000 UV-visible spectrophotometer (NanoDrop Co., USA), the concentration of purified plasmid DNA was adjusted for subsequent DNA sequencing.

3.2.6 Map construction

Genomic DNA was extracted from the leaf tissue of three-week old seedlings, based on a modified CTAB method described by Stein *et al.* (2001). DH lines and two parental varieties were genotyped with DArTSeq (http://www.diversityarrays.com /dart-application-dartseq). Due to the large number of DNA markers (>5,800 SNP and >13,500 DArTSeq markers), markers with removing non-polymorphic and low quality markers were removed from map construction. A total of 8,528 markers were selected to construct the genetic map. The software package JoinMap 4.0 (Van Ooijen and Kyazma, 2006) was used to construct the linkage map.

3.2.7 QTL and statistical analysis

The average values from each experiment were used for the identification of QTL associated with salt tolerance. The software package MapQTL6.0 (Van Ooijen and Kyazma, 2009) was used to detect QTLs which were first analysed by interval mapping (IM). The closest marker at each putative QTL identified using interval mapping was selected as a cofactor and the selected markers were used as genetic background controls in the approximate multiple QTL model (MQM). A logarithm of the odds (LOD) threshold value of 3.0 was applied to declare the presence of a QTL at 95% significance

level. To determine the effects of other traits on the QTL for salinity tolerance, QTL for salinity tolerance were re-analysed by using flowering time and leaf Na/K ratio as covariates. The percentage of variance explained by each QTL (R^2) was obtained using restricted MQM mapping implemented with MapQTL6.0. Graphical representation of linkage groups and QTL was carried out using MapChart 2.2 (Voorrips, 2002). All other statistical analyses, for example, calculation of mean values and standard errors and analysis of variances (ANOVA), were performed using SPSS software package (Version 20.0, IBM).

3.3 Results

3.3.1 Salinity tolerance of parental varieties and DH lines

After one week of treatment the lower leaves of susceptible genotypes started to wilt. In SalinityW treatment, significant differences in leaf yellowing between the two parental varieties became obvious one-week (summer trials) or two-week (winter trials) post treatment. SalinityW stress caused much greater damage than SalinityD stress at the same time period **(Table 3.1)**.

In both SalinityD and SalinityW treatments of the summer trial, TAM704227 showed the most tolerance with a damage score of only 2.0, while Naso Nijo and Gairdner were the most sensitive with scores of greater than 7.0. TX9425 showed very good tolerance to SalinityD treatment (with a score of 2.5 after two-weeks treatment in the summer trial and 3.5 after four-weeks treatment in winter trial) but became sensitive under SalinityW treatment (scored 6.0 after two week treatment in summer trials and 6.3 after three-week treatment in winter trials) (**Table 3.1**; please note that only scoring data for the summer trials are shown). Difference in leaf chlorosis under SalinityD stress (in summer trials) between the two parental varieties YSM1 and Gairdner could be detected after one-week treatment **(Figure 3.1A)** and became significant after four-week treatment **(Figure 3.1B)**.

Table 3.1 Score values of SalinityD/SalinityW tolerance and flowering time for two parental varieties and four control varieties in summer trials.

Variety	2-week treatment		3-week treatment		4- week treatment		Flowering time
	SalinityD	SalinityW	SalinityD	SalinityW	SalinityD	SalinityW	
Gairdner	6	7.5	7	7.75	6	7.5	3
YSM1	3	3	3	3.25	3.5	4	2.25
Baudin	3.25	6.25	3.25	6.25	5.5	5.5	3
TAM704227	2.25	2	1.25	2	1	0.5	1
TX9425	2.5	6	3.5	6.25	5.5	4	2
Naso Nijo	5.75	8.75	7	9	8.5	9	2.83

Figure 3.1 Comparison of salinity tolerance of two parental varieties YSM1 and Gairdner. (A) after 2-week SalinityD treatment and (B) after 4-week SalinityD treatment.

Leaf K^+ content was 2-fold higher in DH lines treated with SalinityD stress (234.20±6.86) than SalinityW stress (110.39±2.58) **(Table 3.2)**. Similarly, the Na/K ratio in DH lines under SalinityW stress (5.24±0.11) was 2-fold higher than those treated

with SalinityD stress (2.13±0.08). However, no significant difference was detected for the leaf Na^+ content in those lines when comparing these two treatments ($p<0.05$). For the two parental varieties, the leaf Na/K ratio were significantly higher (3-5 fold) for YSM1 than Gairdner under both SalinityD and SalinityW treatments. The leaf K^+ retention in both two varieties was more severely repressed by SalinityW stress than SalinityD stress. DH lines showed significant difference in salinity tolerance in both winter and summer trials under drained and waterlogged conditions ($p<0.01$) **(Table 3.3)**. Frequency distributions for plant damage scores were all approximately normally distributed **(Figure 3.2A, 3.2B)**. Significant differences in Na/K ratio also existed among the DH lines under both SalinityD and SalnityW treatments ($p<0.01$) **(Table 3.3)**.

Table 3.2 Comparison of leaf Na content, K content and Na/K ratio of two parental varieties and 122 DH lines under SalinityD and SalinityW treatments.

Genotypes	SalinityD			SalinityW		
	Na^+ content (μM)	K^+ content (μM)	Na/K ratio	Na^+ content (μM)	K^+ content (μM)	Na/K ratio
Gairdner	435.03±28.93[a]	207.92±46.92	2.20±0.66	742.43±53.66	106.83±5.56	7.02±1.37
YSM1	308.68±15.14	351.09±17.81	0.81±0.07	573.23±47.24	124.93±5.53	4.58±0.18
DH lines	468.77±19.93	234.20±6.86	2.13±0.08	551.66±12.66	110.39±2.58	5.24±0.11

[a] Data are mean±SE.

Table 3.3 ANOVA results of 122 DH lines in salinity tolerance under various environments and leaf Na/K ratio in summer and winter trials ($p<0.01$).

Source of Variation	Summer trials						Winter trials					
	Damage scores under SalinityD		Damage scores under SalinityW		Leaf Na/K ratio under SalinityD		Leaf Na/K ratio Under SalinityW		Damage scores under SalinityD		Damage scores under SalinityW	
	MS	F value	MS	F value	MS	F value	MS	F value	MS	F value	MS	F value
Genotype	12.22	7.84**	7.35	3.79**	1.66	1.61**	3.29	1.91**	2.31	1.75**	3.12	2.70**
Replication	12.96	8.31**	42.15	21.77**	0.36	0.35	20.29	11.78**	0.18	0.13	1.73	1.50
Error	1.56		1.93		1.03		1.72		1.32		1.15	

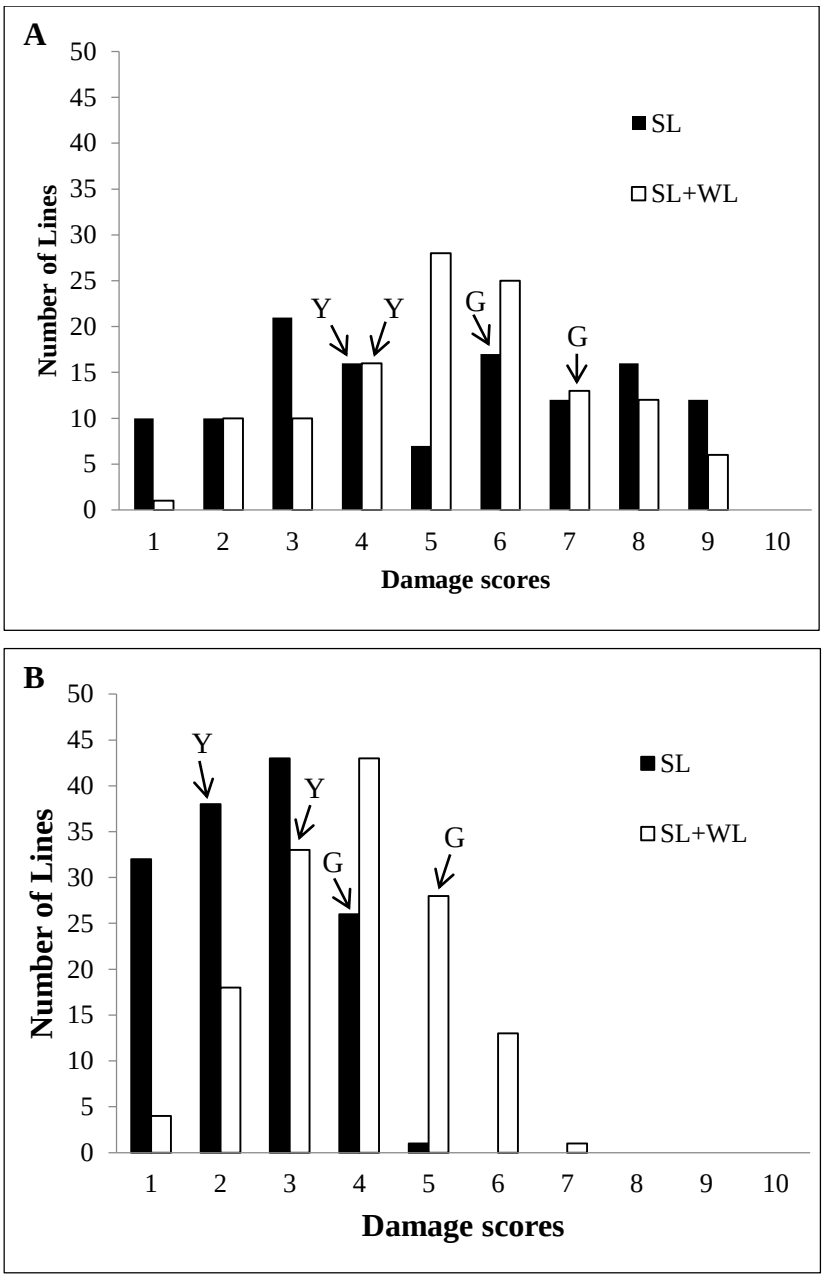

Figure 3.2 Distribution of salinity tolerance (damage scores) of DH lines under drained and waterlogged conditions in summer trials (A) and winter trials (B) (parental varieties included). Solid bars: the number of individuals after four-week SalinityD treatment and hollow bars: the number of individuals after two-week SalinityW treatment. The arrows indicate the scores for YSM1 and Gairdner. 'Y'=YSM1, 'G'=Gairdner.

3.3.2 Evaluation of plant development stages (flowering time) in summer trials

Plant development stages was scored only in the summer trial as the effect of vernalisation genes cannot be clearly detected in winter time. The DH lines were scored as 1 (plants that stayed at tillering stage) to 3 (plants that reached heading stage). The average scores of two parental varieties are 2.25 for YSM1 (stem elongation stage) and 3 for Gairdner (heading stage), respectively **(Table 3.1)**. The distribution for flowering time of DH lines is shown in **Figure 3.3**. There were 48 lines staying at tillering or later than YSM1, while 74 lines were earlier than YSM1. The frequency distribution skewed towards the non-vernalisation-requiring parent Gairdner **(Figure 3.3)**. Among the 48 late-flowering lines, 29 lines required strict vernalisation conditions and remained at tillering stage **(Figure 3.3)**. The salinity tolerance was also compared between three groups of DH lines. In the summer trial, DH lines with flowering time score ranging from 2.6 to 3 were significantly less tolerant to SalinityW stress than lines at tillering stage (1-1.5) (p<0.05). For SalinityD treatment, the significant difference between lines at tillering stage (1-1.5) and those at booting stage (1.6-2.5) or heading stage (2.6-3) was only observed after four-week treatment **(Table 3.4)** (p<0.05).

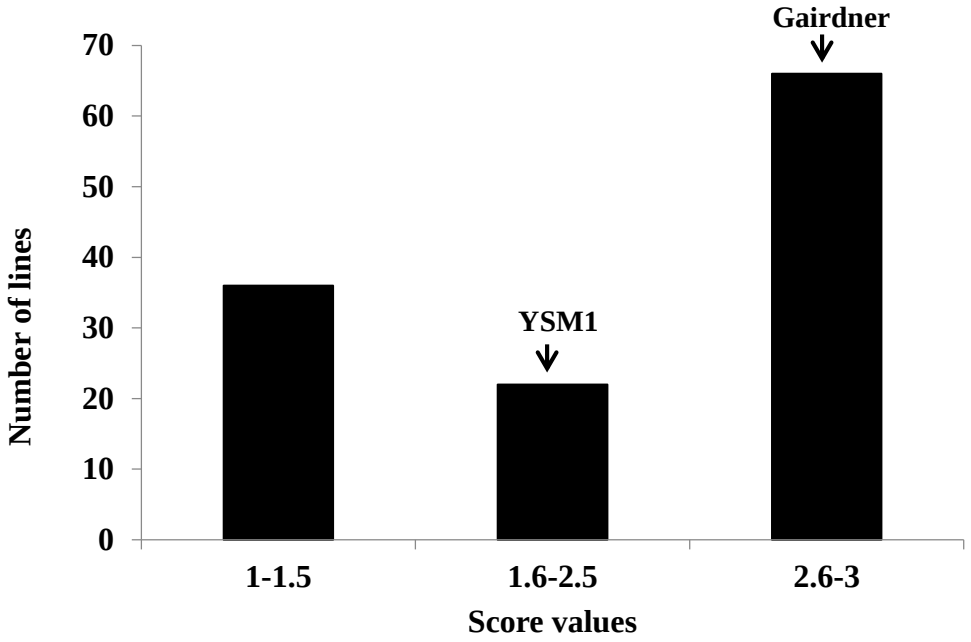

Figure 3.3 Distribution of plant development scores of DH lines after 3-week SalinityD treatment in the summer trial (parental varieties included). The arrows indicate the scores for YSM1 and Gairdner.

Table 3.4 Comparison of SalinityD and SalinityW tolerance (damage scores) in two groups of DH lines differing in flowering time (summer trials).

Score ranges of flowering time	2-week treatment		3-week treatment		4-week treatment	
	SalinityD	SalinityW	SalinityD	SalinityW	SalinityD	SalinityW
1-1.5	3.08	2.88	4.30	4.19	2.88	3.60
1.6-2.5	2.16	4.05	4.78	5.69	5.81	5.13
2.6-3	2.91	4.38	5.30	6.37	6.77	5.90

3.3.3 QTL conferring salinity tolerance and leaf Na/K ratio

In the summer trial, three QTL were identified for SalinityD tolerance **(Table 3.5)**. They are located on the long arm of chromosome 1H, the short arm of chromosome 2H and the long arm of chromosome 5H, respectively **(Figure 3.4)**. These three QTL are designed as *QSlsd.YG.1H*, *QSlsd.YG.2H* and *QSlsd.YG.5H*, where *Sl* represents for

salinity and *sd* for SalinityD treatment in summer trials. These QTL explained a total of nearly 45% of phenotypic variation **(Table 3.5)**. Only two significant QTL, *QSlsw.YG.2H* and *QSlsw.YG.5H,* with sw representing for SalinityW treatment in summer trials, were identified for SalinityW tolerance. These two QTL are located at similar positions to these identified under SalinityD treatment **(Figure 3.4; Table 3.5)**, while the QTL on 1H identified for SalinityD tolerance was not found in SalinityW treatment. For the trait of leaf Na/K ratio, three QTL, *QNaKsd.YG.1H*, *QNaKsd.YG.2H* and *QNaKsd.YG.5H,* were identified for SalinityD tolerance **(Figure 3.4)**. The major QTL *QNaKsd.YG.5H* was located at the position of 130.9 cM on chromosome 5H with LOD value 5.62, explaining 16.0% of the phenotypic variation **(Table 3.5)**. No significant QTL was detected for Na/K ratio under SalinityW treatment.

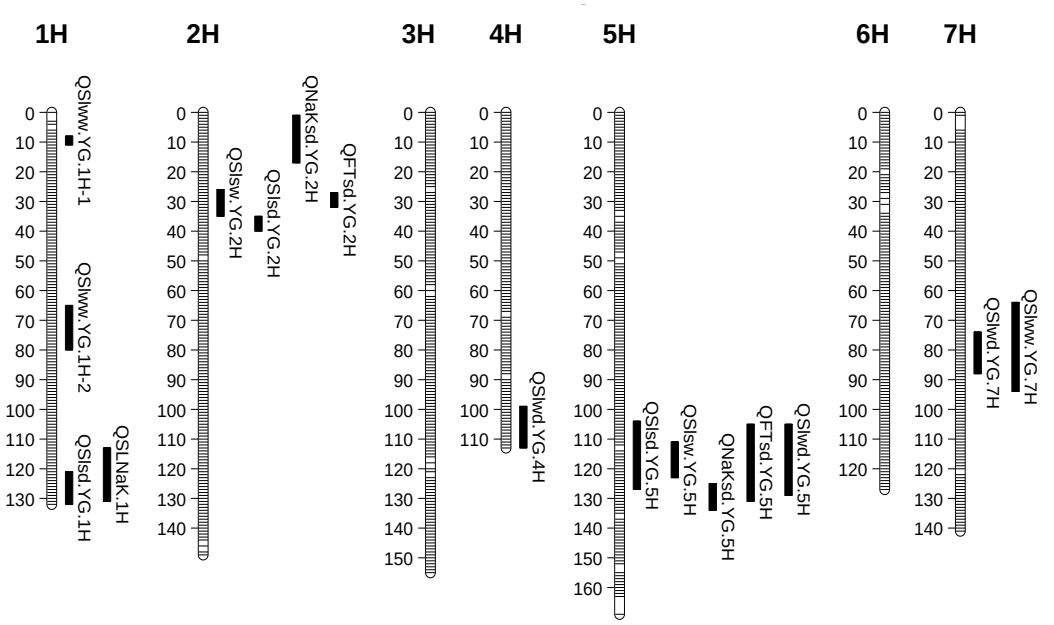

Figure 3.4 QTL associated with salinity tolerance, flowering time and leaf Na/K ratio. Map distances in centiMorgan (cM) are on the left.

In the winter trial, four QTL for SalinityD tolerance, *QSlwd.YG.3H*, *QSlwd.YG.4H*, *QSlwd.YG.5H* and *QSlwd.YG.7H* were identified under drained conditions, with *wd* representing SalinityD treatment in winter trials **(Figure 3.4)**. These four QTL could explain a total of 38% of phenotypic variation **(Table 3.6)**. The major one *QSlwd.YG.7H* is located at the position of 80.4 cM on chromosome 7H with a LOD value of 7.09 **(Figure 3.4; Table 3.6)**. A total of three QTL, *QSlww.YG.1H-1*, *QSlww.YG.1H* -2 and

QSlww.YG.7H (ww representing SalinityW treatment in winter trials), were detected, accounting for 38.2% of phenotypic variation **(Table 3.6)**.

3.3.4 QTL associated with plant flowering time in summer trials

Two QTL, *QFTsd.YG.2H* and *QFTsd.YG.5H,* were identified for plant development, with FT representing for flowering time. The QTL on chromosome 2H (position 31.5 cM) explained 38.0% of phenotypic variation, which shared the same closest maker as *QSlsw.YG.2H* and is 8 cM away from *QSlsd.YG.2H* **(Figure 3.3)**. *QFTsd.YG.5H* with a LOD value of 3.89, was mapped at a similar position as *QSlsd.YG.5H and QSlsw.YG.5H* **(Figure 3.4; Table 3.5)**.

3.3.5 Effects of flowering time on salinity tolerance

DH Lines with late flowering lines showed greater tolerance than lines with early flowering **(Table 3.4)**. QTL associated with plant development genes identified in this study showed significant effects on salinity tolerance in summer trials. The positions of the QTL associated with plant development stages were found to be overlapped with those for salinity tolerance mapped on 2H and 5H. To quantify the effects of plant development stages (flowering time) on SalinityD and SalinityW tolerance, plant development stages was used as a covariate when analysing QTL for salinity tolerance in summer trials. The LOD value of *QSlsd.YG.2H* was significantly reduced from 7.78 to 4.43, with R^2 decreasing from 17.9% to 7.5% when plant development stage was considered **(Figure 3.5A, Table 3.5)**. The QTL *QSlsw.YG.5H* for SalinityW tolerance, became insignificant (LOD<3.0) when flowering time was used as a covariate. Similarly, when plant development stages was accounted for, the LOD value of *QSlsw.YG.5H* decreased from 6.31 to 3.45, while *QSlsd.YG.5H* became insignificant (LOD<3.0) **(Figure 3.5B, Table 3.5)**. Meanwhile, QTL mapped on chromosome 1H (position 129.8 cM) remained highly significant and its position was not affected by flowering time **(Table 3.5)**. Moreover, a new QTL on 1H, *QSLsd.YG.1H-1* with the closest marker being located at 21.7 cM was detected when using flowering time as the covariate **(Table 3.5)**.

Table 3.5 QTL for SalinityD tolerance (SD), SalinityW tolerance (SW), flowering time (FT) and leaf Na/K ratio (SDNaK) identified in the DH population of Gairdner × YSM1 in summer trials (Only QTL with LOD value>3.0 were shown)

Traits	QTL	Linkage Group	Position (cM)	Nearest marker	LOD	R^2 (%)
SD	*QSlsd.YG.1H*	1H	129.8	3665819S[a]	7.06	16.0
	QSlsd.YG.2H	2H	39.5	3260104S	7.78	17.9
	QSlsd.YG.5H	5H	125.5	3258566S	4.40	9.3
SW	*QSlsw.YG.2H*	2H	31.5	3265675D[b]	3.70	10.2
	QSlsw.YG.5H	5H	120.9	3398786S	6.31	18.4
SDNaK	*QNaKsd.1H*	1H	117.6	3398249D	3.35	9.1
	QNaKsd.2H	2H	8.6	3255845S	3.64	9.9
	QNaKsd.5H	5H	130.9	3273502D	5.62	16.0
FT	*QFTsd.YG.2H*	2H	31.5	3265675D	15.21	38.0
	QFTsd.YG.5H	5H	111.7	3398891S	3.89	7.7
FT-adjusted SD	*QSlsd.YG.1H-1*	1H	21.7	3259735D	3.53	5.9
	QSlsd.YG.1H-2	1H	129.8	3256276D	10.62	20.4
	QSlsd.YG.2H	2H	39.5	3260104S	4.43	7.5
FT-adjusted SW	*QSlsw.YG.5H*	5H	120.9	3398786S	3.45	8.8
SDNak-adjusted SD	*QSlsd.YG.1H-1*	1H	21.7	3259735D	2.97	5.8
	QSlsd.YG.1H-2	1H	131.1	3429901D	5.97	13.8
	QSlsd.YG.2H	2H	31.3	3256717S	9.34	23.2

[a] 'S' stands for SNP markers; [b] 'D' stands for DArT markers.

Table 3.6 QTL for SalinityD tolerance (SD) and SalinityW (SW) identified in the DH population of Gairdner × YSM1 in winter trials (Only QTL with LOD value>3.0 were shown).

Traits	QTL	Linkage Group	Position (cM)	Nearest marker	LOD	R^2 (%)
SD	*QSlwd.YG.4H*	4H	103.7	3271960D	3.79	8.1
	QSlwd.YG.5H	5H	120.9	3398786S	4.04	8.7
	QSlwd.YG.7H	7H	80.4	3432693D	7.09	16.1
SW	*QSlww.YG.1H-1*	1H	10.3	3429856D	5.99	15.4
	QSlww.YG.1H-2	1H	72.2	3666342S	4.66	11.5
	QSlww.YG.7H	7H	82.3	3262160S	6.35	13.3

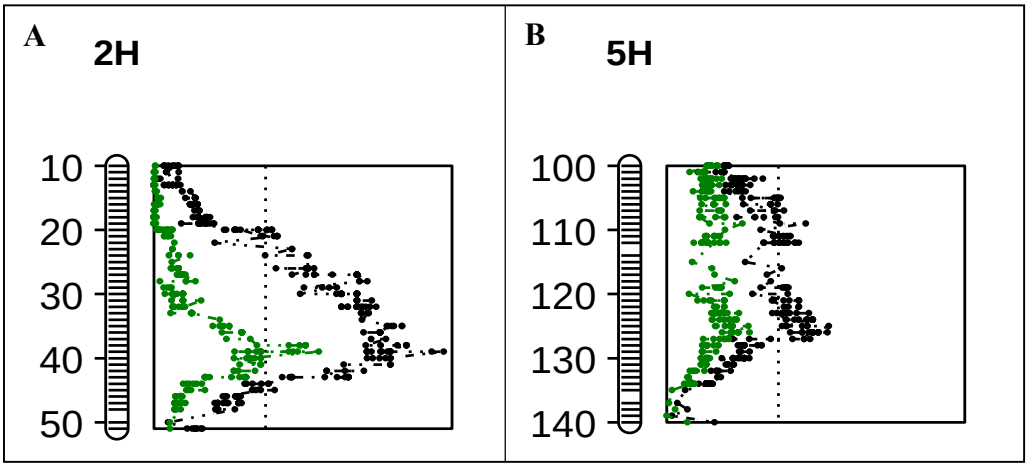

Figure 3.5 Comparison of LOD values of QTL associated with SalinityD tolerance on chromosome 2H (A) and 5H (B) before and after flowering time was used as a covariate. Map distances in centiMorgan (cM) shown to the left. The vertical dotted line indicates the significance threshold of LOD 3.0. Black: LOD value range of original QTL; Green: new LOD value range of QTL when trait of flowering time was used as a covariate.

3.4 Discussion

3.4.1 QTL for salinity tolerance varied under different environments

Many QTL have been reported for salinity tolerance based on various traits, for example, germination rates, chlorophyll content, chlorophyll florescence (Fm/Fv), tissue proline and carbohydrate content, relative water content, coleoptile and radicle length, wet and dry weights of tissues and shoot sodium content (Eleuch et al., 2008, Qiu et al., 2011, Xu et al., 2012, Xue et al., 2010, Zhou et al., 2012). A QTL for Na^+ accumulation was mapped on 1HL using 150 DH lines derived from the cross of Clipper × Sahara 3771 (Lonergan et al., 2009, Rivandi et al., 2011). The locus which is closely linked with the clustered markers, ABC257, cMWG733, BCD808a, and CDO669b, was named as *HvNax4* and explained 79% of the variation in the trait (Rivandi et al., 2011). In another DH population from Harrington×TR306 cross, a single QTL for salinity tolerance at germination stage was also mapped on 1HL with the nearest marker of ABC261. This QTL was located on a similar position to *HvNax4* (Mano and Takeda, 1997, Rivandi et al., 2011). By comparing the above five markers closely linked to *HvNax4* in a barley consensus map (Wenzl et al., 2006) and locating them into a barley physical map, the QTL for salinity tolerance (*QSlsd.YG.1H*) identified in this study is located approximately 20 cM away from the position of *HvNax4*. Further QTL analysis for salinity tolerance using Na/K ratio as a covariate indicated that Na/K ratio showed no significant effects on the QTL mapped on chromosome 1H **(Table 3.5)**. It should be commented though that the whole leaf Na and K analysis fails to account for intracellular sequestration of this ions and, therefore, may not necessarily be causally associated with salinity stress tolerance. This may also indicate that the QTL on chromosome 1H identified in this study is different from *HvNax4.*The two QTL identified on chromosome 1H in the winter trials under waterlogged condition were not found in either the summer or winter trials under drained conditions and no major QTL in these regions were reported before.

Two QTL *QSlsd.YG.2H* and *QSlsw.YG.2H* on chromosome 2H were identified in both drained and waterlogged trials in summer, explaining 17.9% and 10.2% of phenotypic variation, respectively. The position of those QTL is more than 20 cM away from a

major QTL on chromosome 2H for salinity tolerance reported by (Xu et al., 2012). However, *QNaKsd.YG.2H* leaf Na/K ratio identified from the summer trial is very close to that QTL (Xu et al., 2012). In winter trials, no QTL located on chromosome 2H was identified. Instead, a novel QTL for salinity tolerance mapped on chromosome 4H was identified in the winter trial under drained conditions.QTL mapped on chromosome 5H with map interval 120.9-125.5 cM was identified in three of four different environments (salinity treatment in both summer and winter experiments and salinity + waterlogging treatment in summer experiments). The position of this QTL is different from that reported by Zhou *et al.* (2012) but similar to those reported by Siahsar and Aminfar (2010) who mapped a few physiological traits in this region.

QSlwd.YG.7H and *QSlww.YG.7H* identified in the winter trial were located into the same map interval as another Na^+ accumulation QTL, named as *HvNax3*, which is located close to the middle of chromosome 7H and flanked by DArT marker bPb-1209 and microsatellite marker GBM1519 (Shavrukov et al., 2010). Fan *et al.* (2015) also reported a QTL for salinity tolerance in a similar position on chromosome 7H. Those two QTL were also found to be located closely to a QTL identified by Zhou *et al.* (2012) (Zhou et al., 2012) by comparison in the barley consensus map (Wenzl et al., 2006) and barley physical map constructed in this study.

For other QTL identified in the winter trial, *QSlww.YG.1H-2* was found to be mapped closely to a QTL associated with Na/K ratio reported before (Xue et al., 2009). We also found *QSlww.YG.1H-2* was mapped into the same map interval with two QTL for leaf chlorosis and yellowing under two-week and four-week waterlogging respectively, although those two QTL only account for 7.1% and 5% of the phenotypic variation (Li et al., 2008).

3.4.2 Flowering time showed significant effects on plant tolerance to salinity

Two QTL were identified for plant development (trait of flowering time in this study) on 2HL and 5HL, based on plant development stages in the summer trial. Among them, *QFTS.YG.2H* was a major QTL with LOD value of 14.37 and explaining 36.4% of the phenotypic variation. This QTL is at the same position as previously reported photoperiod response locus (*PPD-H1*) (Dunford et al., 2002, Hemming et al., 2008,

Laurie et al., 1994, 1995, Read et al., 2003). The minor QTL on chromosome 5H was mapped to the same region as the major QTL for vernalisation requirements found in the YYXT × Franklin DH population (Wu et al., 2012), which is near *VRN-H1* by comparing the marker BCD265b (von Zitzewitz et al., 2005) using a barley consensus map (Wenzl et al., 2006).

Later flowering or non-flowering DH lines showed generally better tolerance in summer trials **(Table 3.4)**. When using plant development stage as a covariate to analyse QTL for salinity tolerance, the most significant reduction in QTL effects was observed for salinity tolerance QTL on both chromosome 2H and 5H. The LOD value of the QTL on chromosome 2H decreased from 7.78 to 4.43 under drained conditions and from 3.7 to insignificant under waterlogged conditions. Similarly, the LOD value of the QTL on chromosome 5H decreased from 4.4 to insignificant (<3.0) under drained condition and from 6.31 to 3.45 under waterlogged condition. The effect of development genes on stress tolerance has also been reported in many other studies (Wada et al., 2010, Yaish et al., 2011). A zinc finger transcription factor, BBX24 from *Chrysanthemum morifolium* has been identified to be associated with both flowering time and stress tolerance (Hirayama and Shinozaki, 2010). NAC (NAM, ATAF1,2 and CUC2) protein AtRD26 /ANAC072 from *Arabidopsis* and BnNAC485 from *B. napus* play roles in plant flowering time and ABA-mediated pathway in response to abiotic stresses (Ying et al., 2014). At the epigenetic level, KINASE BINDING PROTEIN1 (SKB1) can suppress *FLC* (Flowering Locus C) expression as well as a number of stress-responsive genes, including salt tolerance related genes, by binding to chromatin and increasing the histone methylation level. As a result, the phenotypes with those corresponding epigenetic changes exhibit salt hypersensitivity, late flowering, and growth retardation (Yaish et al., 2011, Zhang et al., 2011). Apart from genetic evidence, the hormone study in *Arabidopsis* showed that jasmonate (JA) can delay flowering occurrence and enhance protection against biotic stress (Chehab et al., 2012). Methyl jasmonate (MeJA) has also been found to act as a vital cellular regulator that mediates diverse developmental processes and defence responses against biotic and abiotic stresses (Cheong and Choi, 2003). Meanwhile, growth and developmental processes of plants can vary in response to stress conditions. During periods of environmental stress, it is common that plant transit to reproductive development earlier than in optimal growth environments (Wada et al., 2010, Yaish et al., 2011). However, there were plants prone to apply the

'quiescence' strategy to conserve the energy without shoot or leaf elongation and use ATP economically when exposed to stresses, for example the low-oxygen quiescence strategy (LOQS) applied by plants under submergence stress (Bailey-Serres and Voesenek, 2008, Colmer and Voesenek, 2009). The 'quiescence' strategy may account for our results that late flowering lines were relatively more tolerant than the early-flowering plants **(Table 3.4)**.

In conclusion, several significant QTL (with LOD>3.0) for salinity tolerance were identified under different environments. Environments showed significant effects on QTL identified for salinity tolerance. The major QTL on chromosome 1H (130.9 cM) *QSlsd.YG.1H* for SalinityD tolerance in the summer trial and the major QTL on chromosome 1H (10.3 cM) *QSlww.YG.1H-1* for SalinityW tolerance in the winter trial were not reported before from other environments. The QTL mapped on chromosome 2H in summer trials were not detected in winter trials while the QTL on chromosome 4H and 7H were only identified in the winter trial. Plant development stage associated locus *QFTsd.YG.2H* and *QFTsd.YG.5H* were found to have significant effects on salinity tolerance, as late flowering lines showed better salinity tolerance, which has provided new clues for understanding the mechanisms for plant tolerance to salinity stress.

Chapter 4 Roots conditioning with hypoxia increase aluminium and acid stress tolerance by mitigating activation of K^+ efflux channels through ROS in barley: insights into cross-tolerance mechanisms [3]

4.1 Introduction

Approximately 30% of total arable lands are acidic (Vonuexkull and Mutert, 1995, Ma and Ryan, 2010). Aluminium (Al) toxicity is the most deleterious factor associated with soil acidity, due to a strong pH-dependence of its solubility (Hoekenga et al., 2003, Wang et al., 2006). Soil acidity occurs mainly in the humid northern temperate zone and the humid tropics (Zhou et al., 2013). Thus, soil acidification could often be closely associated with soil waterlogging in high rainfall regions (Khabaz-Saberi and Rengel, 2010). The duration of waterlogging could be either short-term or long-term, depending on the amount of rain, evapotranspiration and soil structure (Malik et al., 2002). Even short-term/transient soil waterlogging (lasting for only a few hours or days) can have considerable effects on root growth/survival, nutrient uptake and yield of dry land plants (Barrett-Lennard and Shabala, 2013, Malik et al., 2002) Under waterlogged conditions, the main constraints imposed on plants are a shortage (hypoxia) or complete deprivation (anoxia) of oxygen. This results in an energy crisis in roots (Colmer and Voesenek, 2009) and therefore modifies its ability to respond to other environmental constraints. Meanwhile, the significant accumulation of toxic secondary metabolites can occur in waterlogged soils, for example, the phenolic acids, which could become injurious in the low-pH rhizosphere and further reduce the pH values of waterlogged soil (Greenway et al., 2006, Pang et al., 2007, Shabala, 2011). Previous reports have shown that the tolerance of waterlogging stress in barley may be largely determined by the superior ability to reduce the detrimental effects of secondary metabolites (especially for high ability in K^+ retention) in roots (Pang et al., 2007). Only few reports have been focused

[3] This chapter has been accepted as: **Ma YL**, Zhu M, Shabala L, Zhou MX, Shabala S. Root conditioning with hypoxia increases aluminium and acid stress tolerance by mitigating activation of K^+ efflux channels by ROS in barley: insights into cross-tolerance mechanisms. Plant Cell and Physiology, 2016, 57: 160-173

on the interaction between the waterlogging and Al stresses. It was found that waterlogging increases concentrations of Al in acidic soils, resulting in an increase in Al concentrations in the shoot in wheat (Khabaz-Saberi and Rengel, 2010). However, no mechanistic observation for this phenomenon was given.

Both waterlogging and low-pH/Al stresses were found to affect root plasma membrane potential (MP). While the low-pH alone could depolarize MP through rapid H^+ influx into root tissue (Bose et al., 2010a), there is still controversy of whether Al induces depolarization or hyperpolarizition of the plasma membrane (Bose et al., 2010b). In contrast, oxygen deprivation was able to rapidly depolarize the membrane potential (Teakle et al., 2013, Zeng et al., 2014). It has to be mentioned that the functional plasma membrane H^+-ATPase is a key element in maintaining MP, which could also regulate internal pH and membrane energization for the secondary H^+-coupled transport (Duby and Boutry, 2009). As most membrane transporters located on plasma and organelle membranes are voltage-gated and K^+ is the most abundant cation in the cytosol, disturbance to the intracellular K^+ homeostasis is inevitable (Ward et al., 2009, Zeng et al., 2014). Some studies found that Al stress could induce K^+ efflux or reduce K^+ uptake to balance the charge created by the exudation of organic acids (Ma et al., 2001, Osawa and Matsumoto, 2002), while some studies reported an Al-induced decrease in net K^+ efflux (Bose et al., 2010b, Tanoi et al., 2005). Similarly, both hypoxia-induced K^+ leak (Buwalda et al., 1988, Zeng et al., 2014) and K^+ uptake (Teakle et al., 2013, Zeng et al., 2014) were reported. This apparent controversy was explained by high tissue specificity of effects of hypoxia on membrane transport systems (Shabala et al., 2014). At the same time, the plant's ability to retain K^+ is often associated with increased stress tolerance to a broad range of abiotic stresses (Anschutz et al., 2014, Pottosin and Shabala, 2014).

Another possible association between two stresses may be related to production of the reactive oxygen species (ROS). While ROS generation during abiotic stresses could potentially cause oxidative damage to cells, ROS could also function as signal transduction molecules mediating plant responses to pathogen infection, environmental stresses, programmed cell death and different developmental stimuli (Mittler et al., 2004, Torres and Dangl, 2005). Accumulation of H_2O_2 in the apoplast is thought to be involved in acclimation responses of plants to drought and salt stresses, including growth and cell wall strengthening (Miller et al., 2010). Hypoxia stress and the re-entry

of oxygen following drainage can induce ROS accumulation in plant roots (Biemelt et al., 2000, Shabala et al., 2014, Xu et al., 2013). ROS produced by the membrane NADPH oxidase are involved in waterlogging-induced ethylene-controlled adaptation to flooding (Banti et al., 2013, Kreuzwieser and Rennenberg, 2014, Steffens et al., 2013). Internal ROS production can be also triggered by Al (Yamamoto et al., 2002), accompanied by the respiration inhibition and ATP depletion. ROS are known to directly activate a range of Ca^{2+}- and K^+-permeable ion channels (Demidchik et al., 2003, Demidchik et al., 2007, Foreman et al., 2003) thus affect cell ionic homeostasis. Keeping in mind an important role of cytosolic Ca^{2+} as a second messenger in abiotic stress responses (Coelho et al., 2002, Laohavisit et al., 2012), changes in ROS level may therefore mediate interaction between acid soil and waterlogging stresses.

The aim of this work was to understand the physiological basis of interaction between soil acidity and waterlogging stresses in selected barley cultivars, and identify common downstream targets and signal transduction pathways mediating plant adaptation to these environmental constraints. We showed that hypoxia- conditioned roots were less sensitive to low-pH/Al stresses and had higher ability to retain cytosol K^+. The priming effects of hypoxia were mainly conferred through ROS and Ca^{2+} signalling, rather than H^+-associated-MP depolarization or the regulation of the high-affinity K^+ uptake gene *HvHAK1*.

4.2 Materials and Methods

4.2.1 Plant materials and growth conditions

A waterlogging-sensitive barley (*Hordeum vulgare* L) variety Franklin (Zhou et al., 2012) was used in the experiments **(Figure 4.1)**. Seeds were supplied by the Tasmanian Institute for Agriculture (Launceston, Australia). Seeds were surface sterilized with 1.0% commercial bleach for 10 min followed by a 40-min thorough rinse with the tap water. Seeds were then grown hydroponically in the aerated basic salt media (BSM) solution (0.5mM KCl+0.1mM $CaCl_2$, pH=5.6-5.7) in the darkness for three days at 25°C (± 2°C). The 3-d old seedlings were then primed (conditioned) for another 3 days in one of the following solutions: 1) hypoxia solution (BSM containing 0.2% agar bubbled with N_2

for 2h), abbreviated as HC (Hypoxia Conditioned); 2) aluminium solution (BSM containing 5 µM Al and 8mM MES buffer, pH=4.0), abbreviated as AlC (Aluminium Conditioned); and 3) phenolic acid solution (BSM containing 200µM 4-hydroxybenzotic acid), abbreviated as HAC (Hydroxybenzoic Acid Conditioned). The choice of the latter treatment was influenced by the fact that secondary metabolite toxicity is considered to be another (in addition to the lack of oxygen) major constraint affecting plant performance under flooding conditions (Shabala, 2011). Plants grown in aerated BSM solution were used as appropriate controls (abbreviated as NC, non-conditioned). For Al-conditioned seedlings, freshly-prepared BSM solution (pH 4.0) was replaced every 24 hours in each growth container.

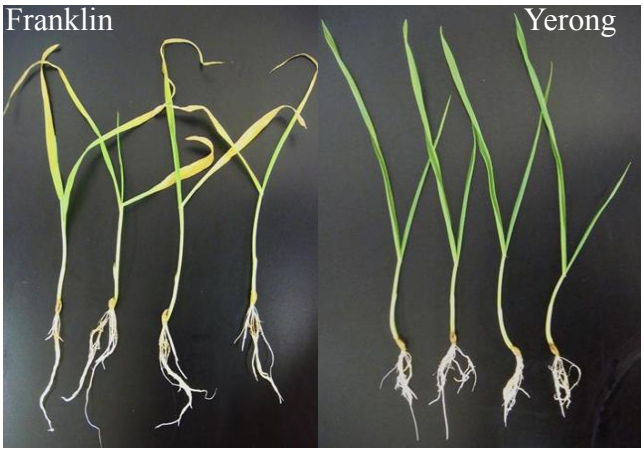

Figure 4.1 Difference in hypoxia stress tolerance between two contrasting barley varieties. Four-day-old seedlings were waterlogged with half-strength Hoagland solution with 0.2% agar for another 10 days with day/night temperature 25±2°C/22±1°C. Variety Franklin is highly sensitive to hypoxia and showed clear symptoms of stress (chlorotic and necrotic leaves). These symptoms are absent in hypoxia-tolerant variety Yerong.

4.2.2 Viability staining

Cell viability was measured using a fluorescein diacetate (FDA; Cat No. F7378; Sigma)-propidium iodide (PI; Cat No. P4864; Sigma-Aldrich) double staining method as described in Koyama et al. (1995). The root apices (~4cm long) and basal root parts (~4cm long from shoot base) were excised and stained with freshly prepared FDA (5 µg ml^{-1}) for 3 minutes followed by PI (3 µg ml^{-1}) staining for 10 minutes. The double

stained root segments were rinsed with distilled water and observed under a florescence microscope (Leica MZ12; Leica Microsystems, Wetzlar, Germany) illuminated by an ultra-high-pressure mercury lamp (Leica HBO Hg 100 W; Leica Microsystems) with an I3-wavelength filter (Leica Microsystems). The excitation and emission wavelengths were 488/505–530 and 543/585 nm for FDA and PI, respectively. Photographs were taken by a camera (DFC295; Leica Microsystems) fitted on the microscope using an image acquisition and processing software LAS V3.8 (Leica Microsystems).

Microscope images were saved as TIFF files and processed for densitometric quantification with ImageJ version 1.47 (NIH). Fluorescence imaging figures were split into different colour channels. FDA is permeable through the intact plasma membrane and internal esterases in viable cells hydrolyse FDA into a green fluorescein (Rotman and Papermaster, 1966), while only dead or dying cells with large pores in the PM incorporate PI and generate red fluorescence (Krishan, 1975). Therefore, the green colour channel excluded with the background colour was chosen for quantifying cell vitality.

4.2.3 Ion flux measurements

Net fluxes of three ions K^+, H^+ and Ca^{2+} were measured from mature and elongation zones of intact barley roots using non-invasive ion-selective vibrating microelectrodes (the MIFE^{TM} technique; University of Tasmania, Hobart, Australia) as described by (Newman, 2001). Microelectrodes were pulled from borosilicate glass capillaries (*GC150-10; Harvard* Apparatus Limited, Kent, UK), overnight oven-dried at 225°C and silanized with chlorotributylsilane (Cat. No. 282707, Sigma-Aldrich). Electrodes with an external tip diameter of 2~3 µm were back-filled with the appropriate solutions (200mM KCl for K^+, 500mM $CaCl_2$ for Ca^{2+} and 15mM NaCl+40mM KH_2PO_4 for H^+) and then front-filled with ionophore cocktails (LIX) as described previously (Shabala and Shabala, 2002). The prepared electrodes were mounted, and calibrated before and after use on a 3D-micromanipulator (MMT-5; Narishige, Tokyo, Japan). Electrodes with response of less than 50mV per decade for monovalent ions (25mV per decade for Ca^{2+}) and correlation less than 0.999 were discarded (Shabala and Shabala, 2002). The microelectrode tips were positioned in one plane with 2-3 µm space between them, with 40 µm above the root surface. During the measurements, the microelectrodes were

moved between two positions 40 µm and 110 µm from the root surface, respectively, by a computer-controlled stepper motor in a square-wave manner (10s cycle). The potential difference between the two positions was recorded by the MIFE CHART software and converted to electrochemical potential difference using the calibrated Nernst slope of the microelectrodes (Shabala et al., 2006). Net ion fluxes were calculated using the MIFEFLUX software for cylindrical diffusion geometry (Newman, 2001).

4.2.4 MIFE experimental protocols

Intact roots of 6-day-old seedlings were taken from growth containers and immobilized horizontally in the measuring chambers filled with either BSM (for NC roots), BSM+0.2% agar (for HC roots), BSM+200µM 4-hydroxybenzotic acid (for HAC roots), or BSM+ 5µM Al (pH 4.0, for AlC roots) solutions and left for about 30min prior to the measurements. Steady-state fluxes were then recorded for 5 min from the respective root zones, before one of the following treatments was given: 1) low-pH (4.0) treatment administered by adding pre-calculated amounts of 0.1N HCl to the bath solution; 2) combined low-pH and Al (5µM of $AlCl_3$) treatment; 3) hypoxia treatment administered by replacing the bath solution with the BSM solution containing 0.2% agar bubbled with N_2; 4) combined hypoxia and low-pH treatment; 5) combined hypoxia, low pH, and Al treatment; and 6) 10 mM H_2O_2 treatment. All measurements were then recorded for another 25 min. The first 1-1.5 min immediately after the treatment were discarded from the data analysis to satisfy MIFE requirements for unstirred layer conditions (Newman 2001) that resulted in a gap as shown in the figures.

4.2.5 Membrane potential measurements

An intact root of a 6-day old barley seedling was mounted in vertical measuring chamber and the longest root was gently secured in a horizontal position with a small plastic block in each experiment. The NC and HC roots were immobilized in either BSM (pH=~5.7) or HCl (0.1N)-added BSM (pH=4.0) for 60 min before the measurements commenced. For MP measurements an Ag/AgCl microelectrode with a tip diameter of ~0.5 µm containing 1M KCl was manually inserted into a root tissue using a micromanipulator (MMT-5; Narishige, Tokyo, Japan) (Cuin and Shabala, 2005).

Changes in the MP values were monitored continually in a computer screen and recorded by the MIFE CHART software (Shabala et al., 2006). Steady MP values were measured from 5 to 6 seedlings for each treatment and at least 5 cells were impaled in each root zone.

4.2.6 Quantitative Real-Time PCR

Root apices (5~7mm long) were collected from 20 seedlings for each treatment. Total RNA was isolated by RNeasy^{TM} plant kit (Qiagen) and purified by RNase-free DNase I (the Isolate II plant RNA kit, Cat. No. BIO-52076, Bioline) and Isolate II micro clean-up kit (Cat. No. BIO-52080, Bioline). Reactions with a 20 μl-volume containing 800 ng total RNA were performed to synthesize cDNA by SensiFAST cDNA Synthesis Kit (Cat. No. BIO-65053, Bioline). Quantification real-time polymerase chain reactions (RT-PCR) were run at Rotor-Gene^{TM}3000 Thermal Cycler (Qiagen) with 10 μl reaction mixture containing 4.2 μl of diluted cDNA samples, 5 μl of SensiFAST SYBR No-Rox mix (Cat. No. BIO-98002, Bioline) and 0.8 μl primer mix (1:1 mix of forward and reverse primers at 10 nM). Primer sequences can be found in the **Table 4.1**. Cycling conditions were as follows: 3 min at 95°C, followed by 45 cycles at 95°C for 5 sec, 60°C for 10 sec and 72°C for 5 sec. At the end, a melting curve of the amplified fragments was produced by increasing the temperature every 1°C from 55°C to 99°C (with a 5s-hold at each temperature). Measurements were repeated three times, with consistent results.

Table 4.1 Primers used in the gene expression study

Gene Name	Forward Primer	Reverse Primer
HvPMHA	5'-GCTGGTGTTATCTGGCTCTTC-3'	5'-CTCTTCTCTTGGCTT GCTCAG-3'
HvHAK1A	5'-GCTGGCCATTCCCAAAGA-3'	5'-GACAATGGCACTTTGCCCTC-3'
HvGAPDH	5'-GTGAGGCTGGTGCTGATTACG-3'	5'-TGGTGCAGCTAGCATTTGAGAC-3'

4.2.7 Statistical analysis

Significant difference between means was evaluated by Student's t-test and ANOVA analysis using a statistical package IBM SPSS Statistics 20(IBM, New York, NY, USA). All data in the figures are shown as means ±SE.

4.3 Results

4.3.1 Root conditioning with hypoxia improves cell viability under low-pH and combined low-pH/Al stresses

Three days of hypoxia conditioning induced some loss of cell viability (reduced intensity of the green fluorescence signal) in the root apex **(Figure 4.2C)** but not in the mature zone **(Figure 4.2D)** compared with the appropriate controls **(Figure 4.2A, 4.2B)**. However, the adverse effects caused by low-pH and combined low-pH/Al stresses were substantially alleviated by the hypoxia conditioning of the root, both in the apex **(Figure 4.2G, 4.2K)** and the mature **(Figure 4.2H, 4.2L)** zones.

To quantify the above ameliorating effect of hypoxia conditioning on acid stress tolerance, the densitometric analysis was conducted in four different root zones: the meristem zone, transition zone, elongation zone, and mature zone (**Figure 4.3**). In the meristem zone the significant effect of hypoxia on cell viability could be detected between non-conditioned (NC) and hypoxia-conditioned (HC) roots in control conditions, but no significant (P<0.05) difference was found between HC and NC roots treated with either low pH or combined low-pH/Al treatments **(Figure 4.3A)**. In the transition zone, priming effects of hypoxia on cell viability merely became significant when roots were exposed to the combined low-pH/Al stress **(Figure 4.3B)**. In contrast, in both elongation zone (EZ) and mature zone (MZ) the extent of damage caused by both low-pH and combined low-pH/Al stresses was more pronounced in NC roots than in HC roots **(Figure 4.3C, 4.3D)**. The combined low-pH/Al stress was always more detrimental than low-pH stress alone.

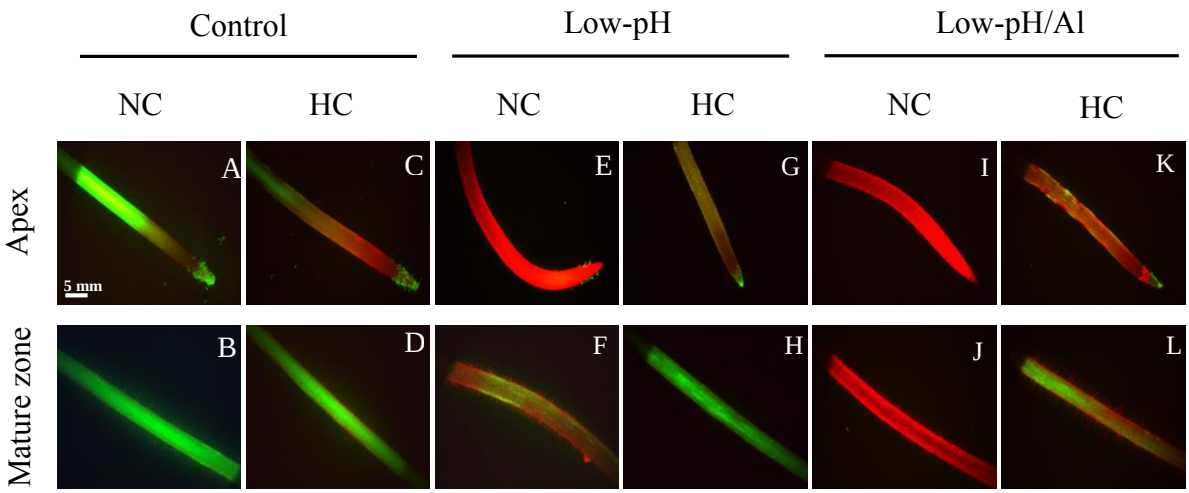

Figure 4.2 Barley root viability after 24 h of low-pH (4.0) and combined low-pH/5µM Al treatments visualised by FDA-PI staining in non-conditioned (NC) plants and plants pre-treated with hypoxia for 3 days (N_2-bubbled 0.2 % agar treatment; labelled as HC). Fluorescence images were taken at the root apex (top panels) and in mature root zone, 10 to 20 mm from the shoot base (bottom panels).

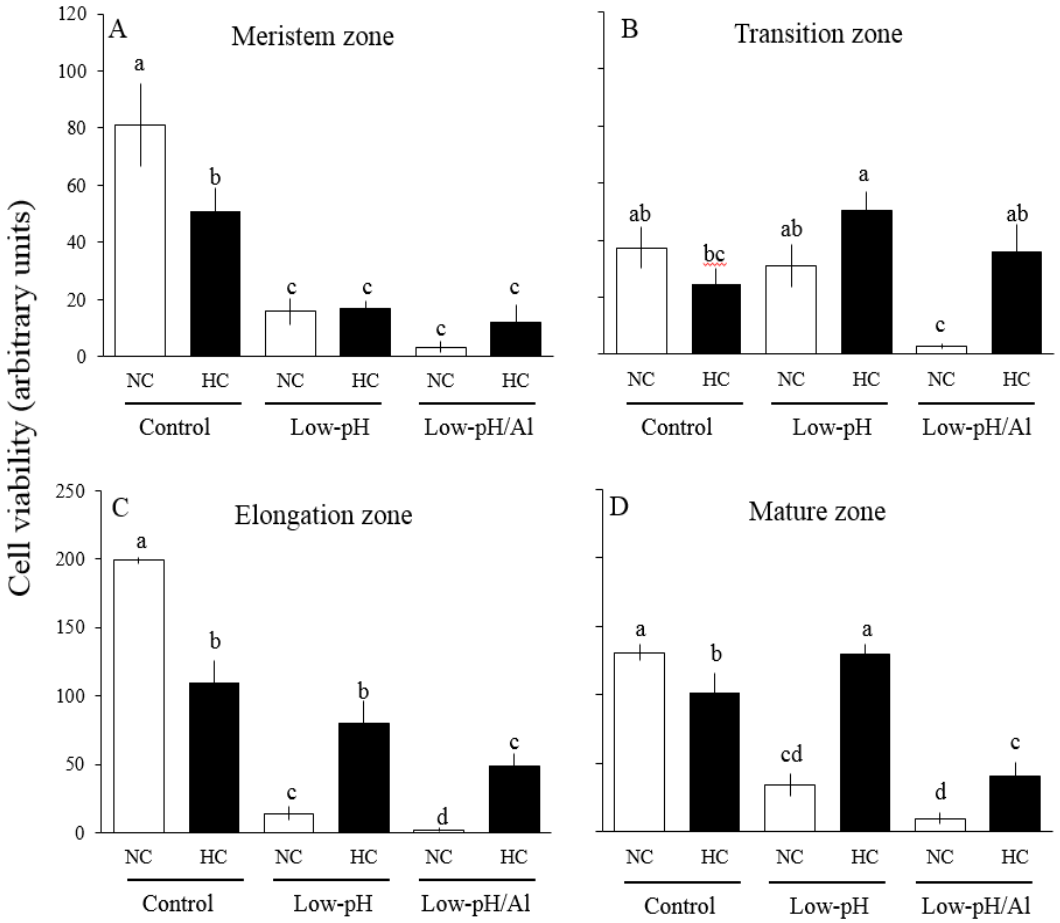

Figure 4.3 Quantification of the cell viability of the root apex. The intensity of green fluorescence signal (a measure of cell viability) was quantified by ImageJ software separately for root meristem zone (A), transition zone (B), elongation zones (C), and mature root zone (D) for six different treatments depicted in Fig 1. In each root zone, treatments sharing the same letter were not significantly different (P<0.05). Data are mean ± SE (n = 4-13 individual roots).

4.3.2 Hypoxia pre-treatment alters root ion flux responses to low-pH and combined low-pH/Al treatments

Based on the results of viability staining, elongation (EZ) and mature (MZ) zones were selected for measuring transient K^+, H^+ and Ca^{2+} fluxes upon short-term exposure to the low-pH and combined low-pH/Al stresses.

Both low-pH and combined low-pH/Al stresses induced transient net K^+ efflux from both EZ and MZ, which decreased gradually over the time (**Figure 4.4**). In the EZ, HC roots exhibited significantly higher ability for K^+ retention than NC when exposed to either low-pH or combined low-pH/Al treatments **(Figure 4.4A, 4.4B)**. The K^+ flux changed from efflux to influx for HC roots after ~6 min of both treatments, while the NC roots maintained K^+ efflux during the entire 30 min measuring period. Effects of low-pH and combined low-pH/Al stresses on K^+ flux kinetics were similar. In the MZ, the priming effects of hypoxia in K^+ retention can only be observed in the treatment of the combined low-pH/Al stress **(Figure 4.4C, 4.4D)**.

Both low-pH and combined low-pH/Al stresses induced dramatic net H^+ influx in both EZ and MZ with magnitude being 2-fold higher in the EZ than the MZ (**Figure 4.5**). The combined low-pH/Al stress induced significantly higher H^+ influx than low pH stress itself in the EZ **(Figure 4.5A)**. Although there was no significant difference in H^+ flux changes in the EZ between HC and NC roots when treated with either the low-pH or combined low-pH/Al stress, NC roots generally showed higher H^+ influx than HC roots from the onset to ~4 min of the two treatments (**Figure 4.5A, 4.5B**). In contrast, the H^+ influx of HC roots was significantly higher than NC roots within the initial 4 min of the treatments in the MZ **(Figure 4.5C, 4.5D)**.

Both low-pH and combined low-pH/Al stresses induced net Ca^{2+} efflux of the same magnitude from both EZ and MZ, which decreased gradually over time **(Figure 4.6)**. A significant difference was observed between NC and HC roots under low-pH treatments in both EZ and MZ (**Figure 4.6A, 4.6C**), with net Ca^{2+} efflux from NC roots being significantly higher than that from HC roots (**Figure 4.6B**). There was little difference between NC and HC roots in both EZ and MZ when exposed to the combined low-pH/Al stress (**Figure 4.6B, 4.6D**).

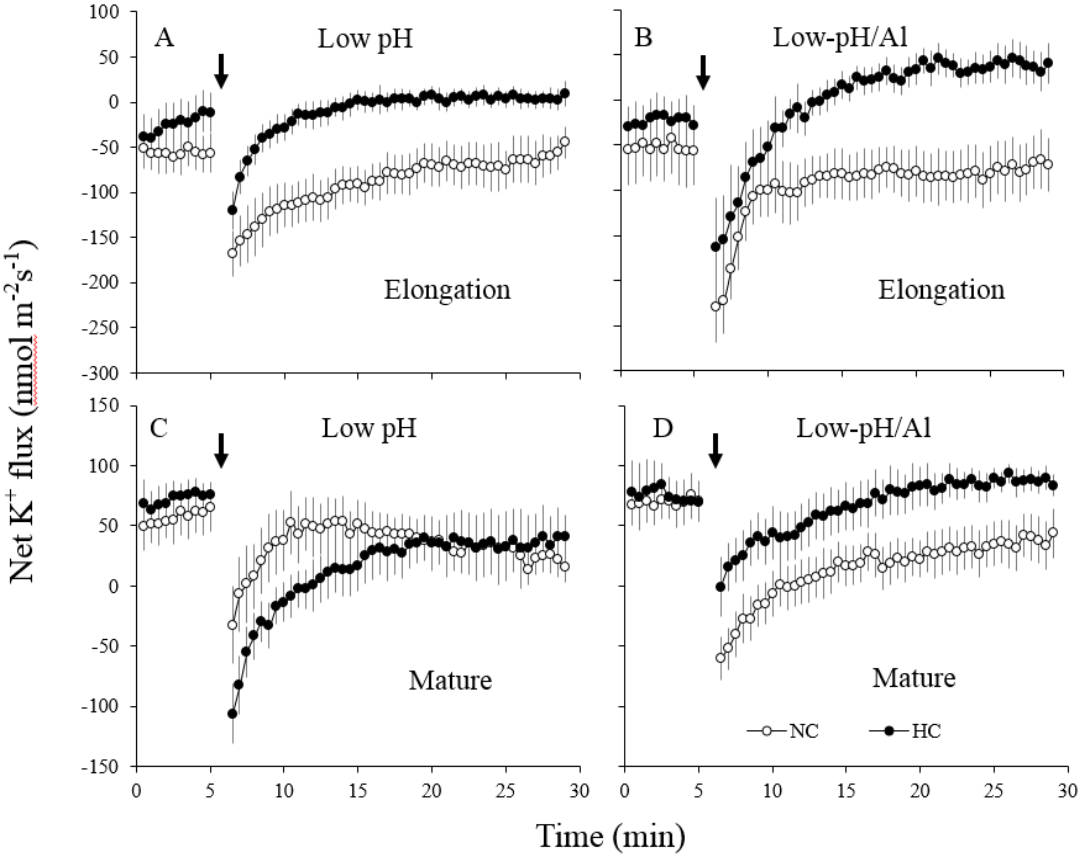

Figure 4.4 Effect of hypoxia conditioning (3 days in N_2-bubbled 0.2 % agar) on net K^+ fluxes measured from barley root epidermis in response to low-pH (4.0) and combined low-pH /5μM Al stresses given at 5 min (as indicated by an arrow). Measurement was taken at the elongation (A, B; 3 to 4 mm from the root cap) and mature (C, D; 10 to 20 mm from the shoot base) zones of 6-day old barley seedlings. The low pH and combined low pH treatments were imposed at time point of 5min. Data are mean ± SE (n=6-8 individual roots). NC, non-conditioned; HC, hypoxia conditioned.

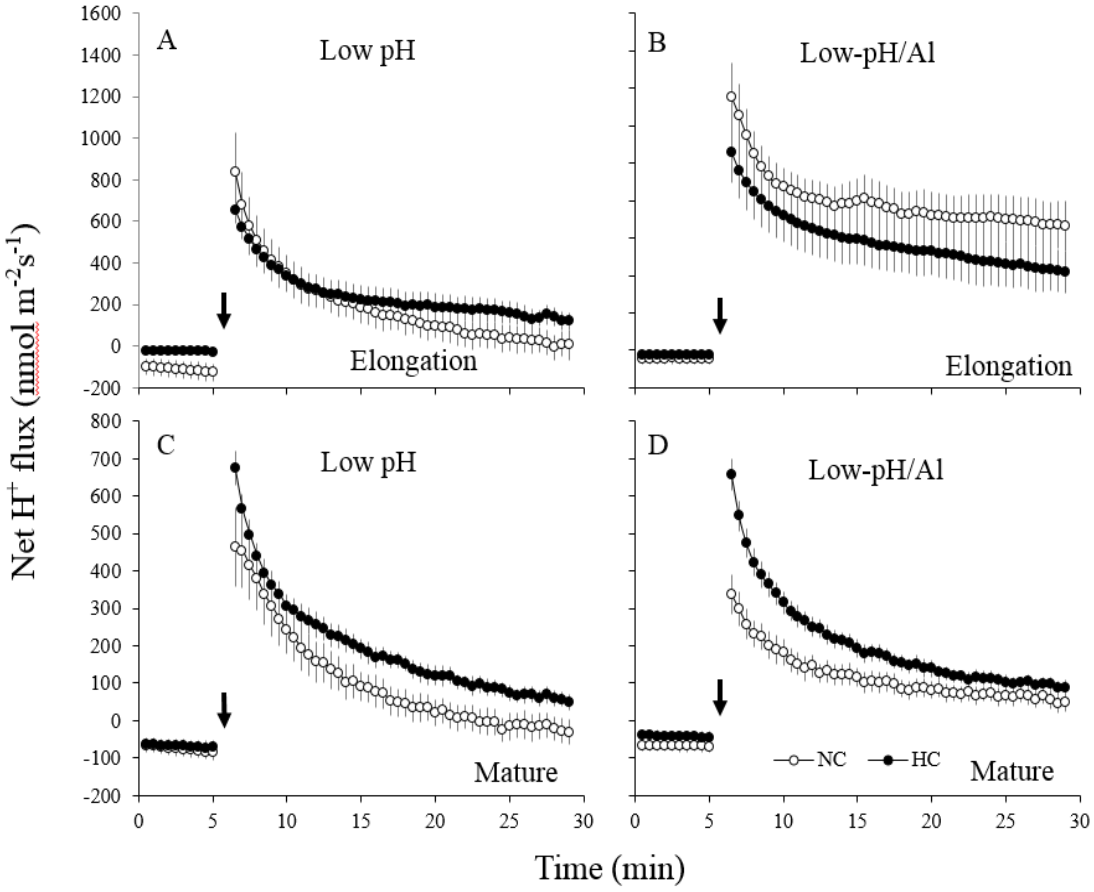

Figure 4.5 Effect of hypoxia conditioning (3 days in N_2-bubbled 0.2 % agar) on net H^+ fluxes measured from barley root epidermis in response to low-pH (4.0) and combined low-pH /5μM Al stresses given at 5 min (as indicated by an arrow). Measurement was taken at the elongation (A, B; 3 to 4 mm from the root cap) and mature (C, D; 10 to 20 mm from the shoot base) zones of 6-day old barley seedlings. The low pH and combined low pH treatments were imposed at time point of 5min. Data are mean ± SE (n = 7-11 individual roots). NC, non-conditioned; HC, hypoxia conditioned.

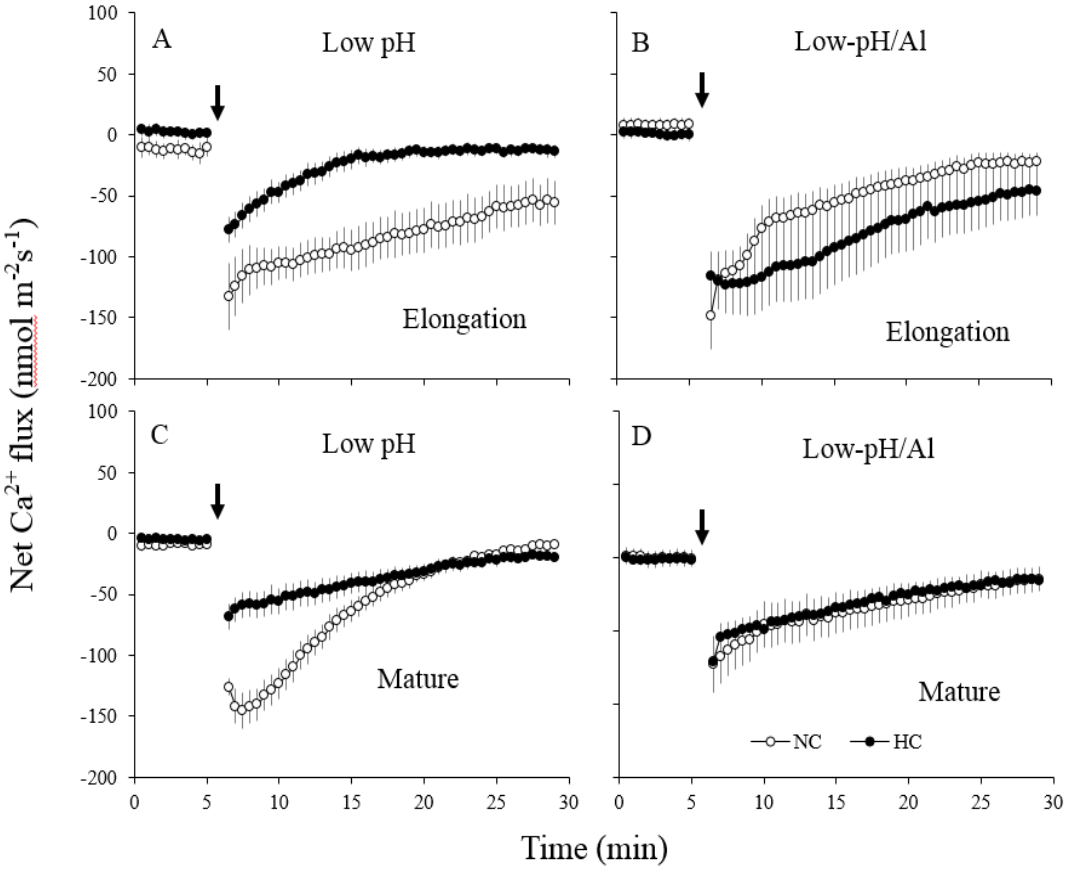

Figure 4.6 Effect of hypoxia conditioning (3 days in N_2-bubbled 0.2 % agar) on net Ca^{2+} fluxes measured from barley root epidermis in response to low-pH (4.0) and combined low-pH /5μM Al stresses given at 5 min (as indicated by an arrow). Measurement was taken at the elongation (A, B; 3 to 4 mm from the root cap) and mature (C, D; 10 to 20 mm from the shoot base) zones of 6-day old barley seedlings. The low pH and combined low pH treatments were imposed at time point of 5min. Data are mean ± SE (n = 6-11 individual roots). NC, non-conditioned; HC, hypoxia conditioned.

4.3.3 Conditioning with 4-hydroxybenzoic acid affects root responses to low-pH/Al stress

4-hydroxybenzoic acid is a secondary metabolite from phenolics group that is accumulated in plant roots under flooded conditions (Shabala, 2011). Here we have tested whether roots conditioning with 4-hydroxybenzoic acid had any impact on tolerance to Al stress. In both EZ and MZ, NC roots displayed higher magnitude of changes in K^+, H^+ and Ca^{2+} fluxes than roots conditioned with 4-hydroxybenzoic acid, when exposed to the combined low pH/Al stress **(Figure 4.7)**. The magnitudes of flux changes in K^+, H^+ and Ca^{2+} at the onset of the combined low pH/Al treatment were significantly higher in the EZ than in MZ. Hydroxybenzoic acid conditioned (HAC) roots exhibited higher ability to retain K^+ and had reduced Ca^{2+} efflux compared with NC roots in both EZ and MZ when treated with low-pH/Al **(Figure 4.7A, 4.7B, 4.7E, 4.7F)**.

4.3.4 Effect of low-pH/Al conditioning on root response to hypoxia

We have also checked whether roots conditioning with Al may have an effect on their ability to respond to hypoxia (e.g. reversing the "conditioner" and "stressor"). Net influxes of H^+ and Ca^{2+} were observed in Al-conditioned (AlC) roots at the start of hypoxia treatment in both EZ and MZ, while net effluxes of H^+ were observed in NC roots in both zones **(Figure 4.8C, 4.8D)**. In the EZ, hypoxia induced net K^+ efflux at the beginning of the treatment which gradually decreased and changed to net K^+ influx at the end of the measuring period **(Figure 4.8A)**. The magnitude of K^+ flux changes in AlC roots was significantly higher in NC roots. In contrast, in the MZ, no significant difference was observed in K^+ flux kinetics between NC and AlC roots **(Figure 4.8B)**. A small net Ca^{2+} influx was observed in NC roots **(Figure 4.8F)**. Unlike the priming effects of hypoxia, conditioning in low-pH/Al pre-treatment only showed beneficial effects on hypoxia tolerance (higher K^+ retention ability) in the EZ but not MZ.

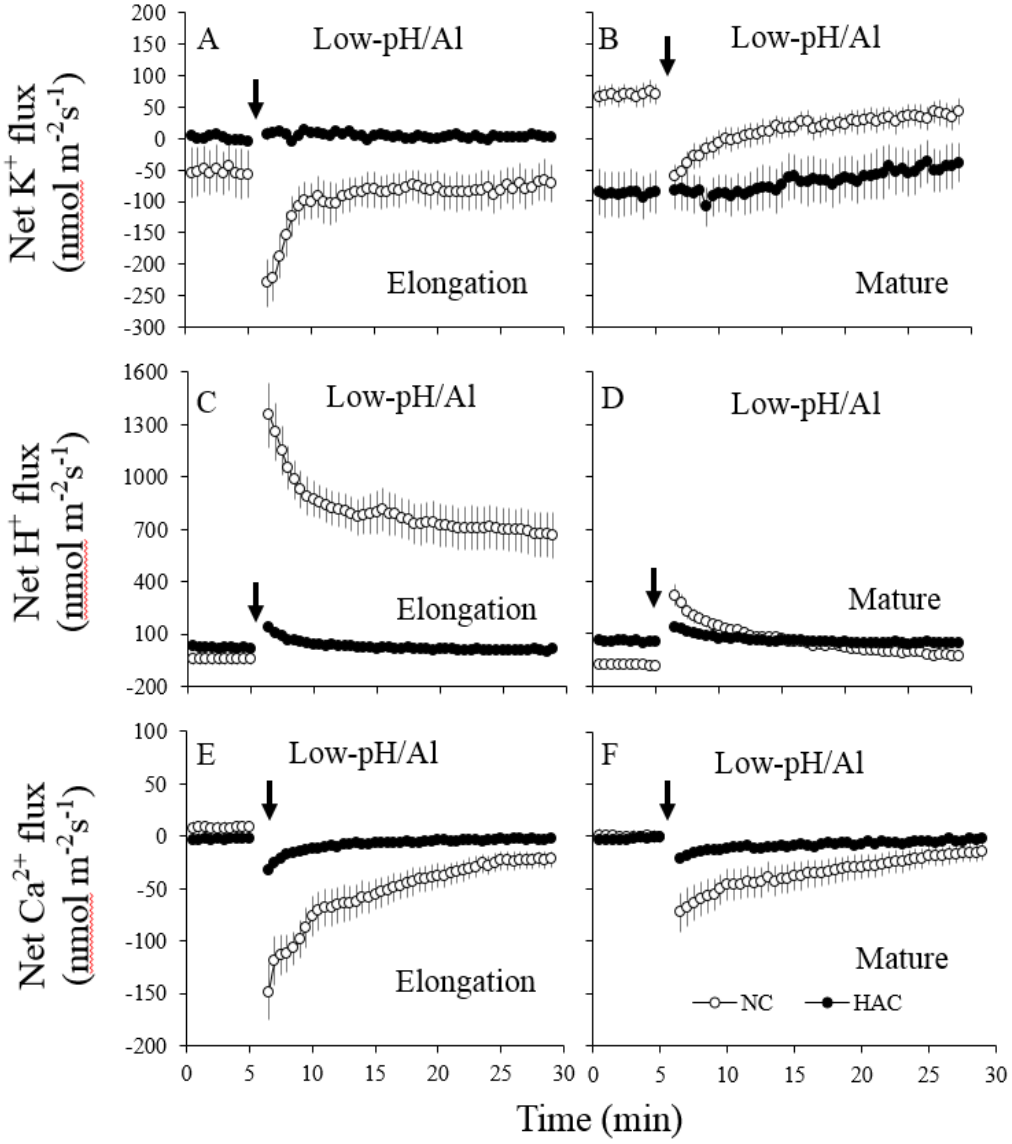

Figure 4.7 Effect of root conditioning with 4-hydroxybenzoic acid on net ion flux responses measured from barley root epidermis upon combined low-pH/Al stress (given at 5 min as indicated by an arrow). Measurement was taken at the elongation (3 to 4 mm from the root cap) and mature (10 to 20 mm from the shoot base) zones. Data are mean ± SE (n=8-11 individual roots). NC, non-conditioned; HAC, hydroxybenzoic acid-conditioned.

4.3.5 Priming effects of hypoxia on K^+ retention are not related to the maintenance of membrane potential or high-affinity K^+ uptake

Stress-induced K^+ efflux is often associated with the plasma membrane (PM) depolarization and activation of depolarization-gated K^+ efflux channels (reviewed in Pottosin and Shabala 2014). Therefore, we further investigated whether the above beneficial effects of hypoxia conditioning on K^+ retention **(Figure 4.4A, 4.4B; Figure 4.8A, 4.8B)** contributed to better membrane potential (MP) maintenance.

In the EZ, the PM was significantly depolarized after 3-day hypoxia conditioning compared with the non-hypoxia treatment **(Figure 4.9A)**. This was consistent with the significantly lower steady-state H^+ efflux observed in HC roots **(Figure 4.5A)**. When exposed to the low-pH stress, NC roots demonstrated ~33% of depolarization compared with ~11% for HC roots. Yet, the absolute MP values were less negative in HC roots compared with NC roots under low-pH stress **(Figure 4.9A)**, ruling out better MP maintenance as a reason for better K^+ retention in HC roots. In the MZ, no significant depolarization was observed after 3-day hypoxia treatment. When exposed to the low-pH stress, no significant depolarization was detected from NC roots while slight depolarization (~11%) was observed in the HC roots ($P<0.05$). The reduced MP in HC roots were consistent with significant (5-fold) down-regulation of H^+-ATPase pump expression (*HvPMHA* gene; the barley homologue of *AHA1/2/3*) **(Figure 4.9B)**.

Further experiments were conducted to see whether smaller net K^+ efflux in hypoxia-conditioned roots may result from increased high affinity K^+ uptake. Unexpectedly, the relative expression of the high-affinity K^+ transporter gene *HvHAK1A* (the barley homologue of *AtHAK5*) was significantly reduced by ~2-fold upon 3-day hypoxia conditioning **(Figure 4.9B)**. Meanwhile, because of the high homology between HvHAK1B and HvHAK1A (93% on predicted amino acid sequences), the function for HvHAK1B in the uptake of K^+ could be hypothesized from HvHAK1A (Vallejo et al., 2005).

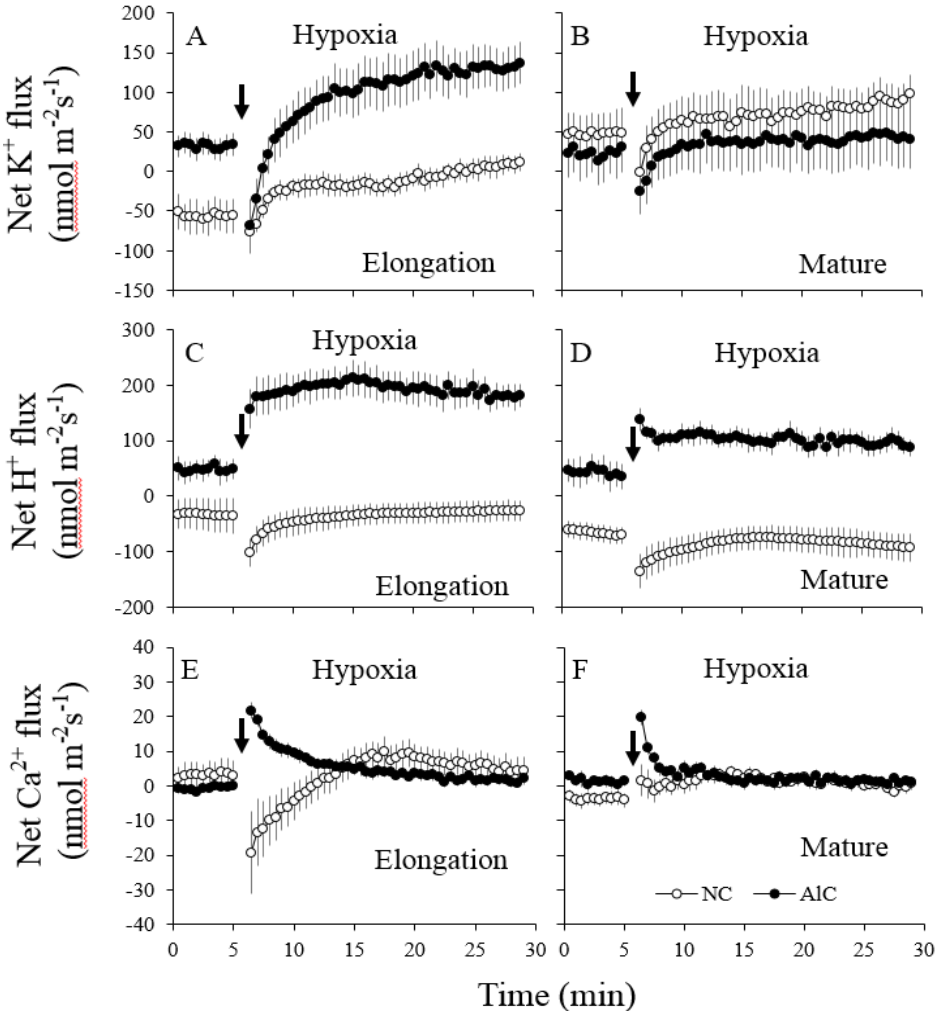

Figure 4.8 Effect of root conditioning with low-pH/Al treatment on net ion flux responses measured from barley root epidermis upon hypoxia stress (0.2% agar bubbled with N_2 given at 5 min as indicated by an arrow). Measurement was taken at the elongation (3 to 4 mm from the root cap) and mature (10 to 20 mm from the shoot base) zones. Data are mean ± SE (n = 6-8 individual roots). NC, non-conditioned; AlC, Al conditioned.

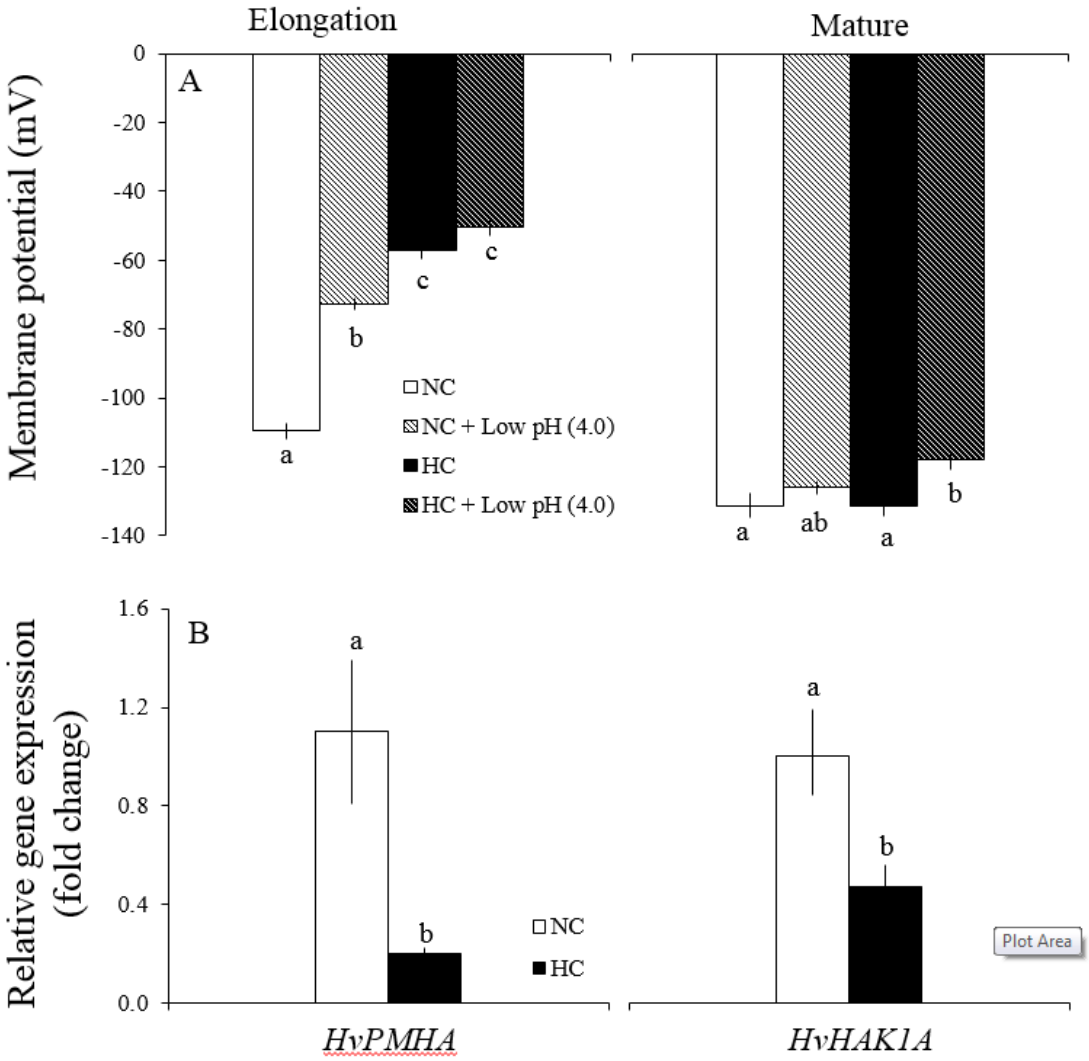

Figure 4.9 A - Effect of hypoxia conditioning (3 days in N_2 – bubbled 0.2% agar) on membrane potential of barley roots treated with low-pH (4.0). Six-day-old seedlings were immobilised in measuring chambers filled with basal salt medium (BSM; 0.5mM KCl +0.1mM $CaCl_2$) at either pH 5.6 (control) or pH 4.0 (low pH treatment) for 60 min before each measurement. Data are mean ± SE (n= 25-35 cells from 5 to 6 individual roots) (P<0.05). **B – changes in a relative expression of barley plasma membrane H^+-ATPase gene (*HvPMHA*) and high affinity K^+ transporter gene *HvHAK1A* in barley root apices (5-7 mm long) after 3-day-hypoxia conditioning**. Data are mean ± SE (n=5-6). For both genes, the observed reduction in gene expression was significant at P<0.05.

4.3.6 Hypoxia conditioning reduces sensitivity of root membrane transporters to ROS

Another possible pathway for stress-induced K^+ efflux may be mediated by ROS-activated K^+ permeable channels (Pottosin and Shabala, 2014). To check if this scenario is applicable to the cross-tolerance between hypoxia and Al stresses, the sensitivity of root ion fluxes to ROS stress applied as 10 mM H_2O_2 has been tested.

In the EZ, exogenous application of 10 mM H_2O_2 induced significantly higher K^+ efflux from epidermal root cells; this efflux was about 10-fold higher in NC compared with HC roots **(Figure 4.10A)**. For NC roots, the K^+ efflux developed gradually and reached peak values after ~10min of the treatment. A similar pattern of ROS-induced K^+ efflux was also observed in roots of barley genotypes with contrasting salt-tolerance (Chen et al., 2007, Maria Velarde-Buendia et al., 2012) as well as roots of *Arabidopsis* (Cuin and Shabala, 2007). Consistent with previous reports (Maria Velarde-Buendia et al., 2012), H_2O_2 treatment resulted in transient Ca^{2+} flux changes; with magnitudes being significantly higher in NC roots than in HC roots **(Figure 4.10C)**. Upon H_2O_2 treatment, net Ca^{2+} flux firstly exhibited efflux, then changed to influx after 7~8 min of the treatment, reaching its peak after ~10min, and remained as net influx and higher than the initial level. The extracellular application of H_2O_2 caused net influx of H^+ in both NC and HC roots with the magnitude of flux being significantly smaller in HC roots **(Figure 4.10)**.

In the MZ, no significant difference in net K^+ loss or H^+ uptake induced by H_2O_2 was observed between the NC and HC roots **(Figure 4.10B, 4.10D)**. Although NC roots displayed higher Ca^{2+} influx than HC roots in the presence of H_2O_2, the difference can be negligible when taking the initial flux values into consideration **(Figure 4.10F)**.

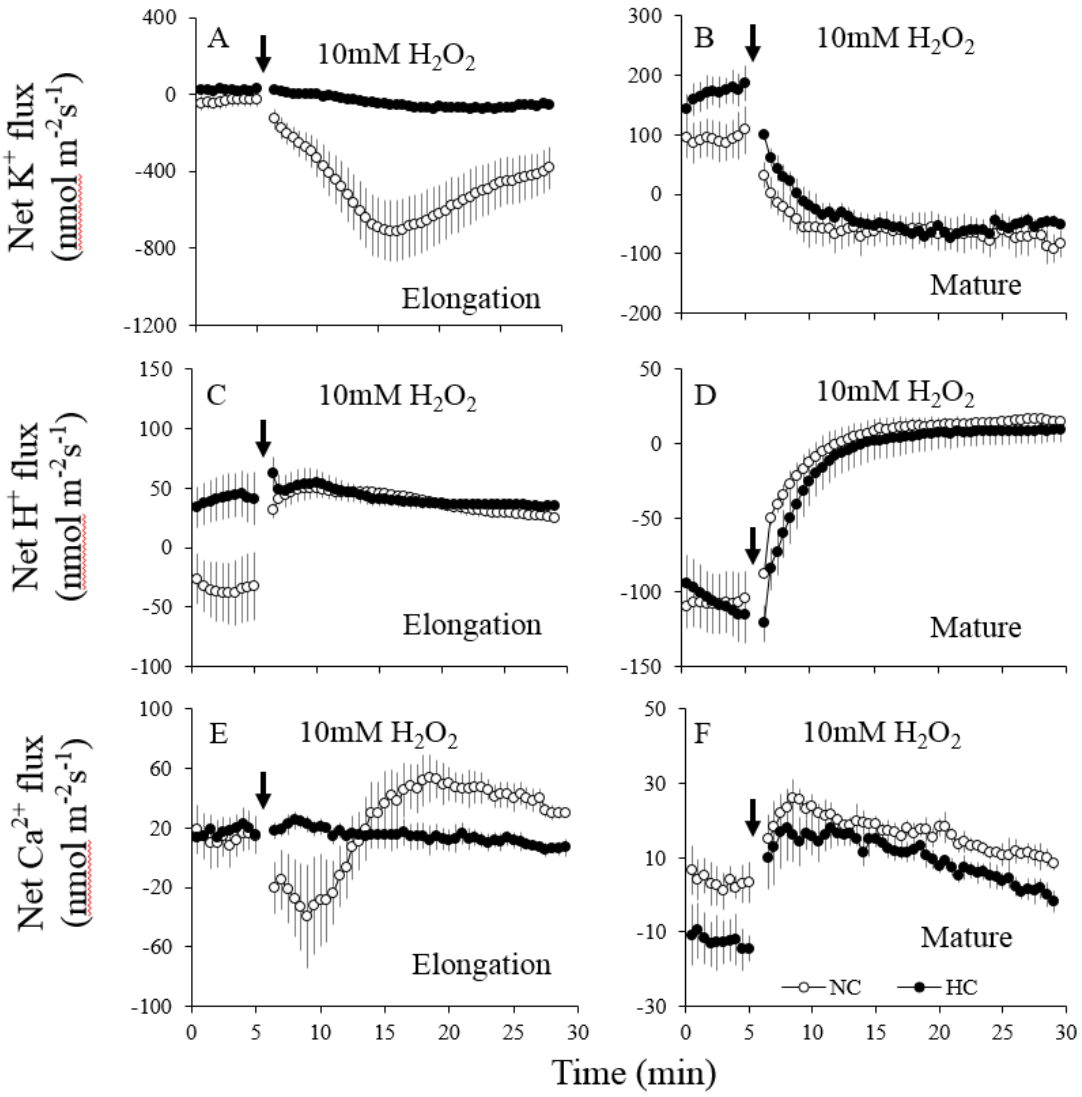

Figure 4.10 Effect of hypoxia conditioning (3 days in N_2 –bubbled 0.2% agar) on root net ion flux response to 10mM H_2O_2 (given at 5 min as indicated by an arrow). Measurement was taken at both elongation zone and mature zone as described above. Data are mean ± SE (n = 5-7 individual roots). NC, non-conditioned; HC, hypoxia conditioned.

4.4 Discussion

This chapter reports, for the first time, the phenomenon of cross-protection between hypoxia and low-pH/Al stresses, and causally links it to cell's ability to maintain cytosolic K^+ homeostasis. We showed that both hypoxia- and phenolic-acid-conditioned barley plants (as under flooding conditions) possess higher ability to retain K^+ in response to low pH/Al stresses. These priming effects were not related to higher H^+-ATPase activity and better membrane potential maintenance, or to the increased high-affinity K^+ uptake by roots but were conferred by desensitization of ROS-permeable K^+ efflux channels. Supporting arguments are given below.

4.4.1 The priming effects of hypoxia are not related to either MP maintenance changes or increased high affinity K^+ uptake by HvHAK1

It has been reported that the ability of roots to retain K^+ is strongly correlated with waterlogging stress tolerance in barley (Zeng et al., 2014). Three factors are shown to be associated with the hypoxia-induced K^+ loss: 1) stress-induced membrane depolarization, 2) changes in non-specific membrane permeability to K^+, and 3) ROS activation of K^+ permeable channels (Shabala et al., 2014). Consistent with previous reports (Zeng et al., 2014), the MP was significantly depolarized after 3-day hypoxia treatment **(Figure 4.9A)** in the root EZ, which was further proved by the supressed production of PM H^+-ATPase transcript in our study (**Figure 4.9B**). However, when exposed to the low-pH stress, the HC roots did not show higher ability to maintain MP values (more negative than NC roots) either in the EZ or MZ **(Figure 4.9A).** These results are not consistent with the cell viability data **(Figure 4.2)** and strongly suggest that the priming effects of hypoxia could not be explained by regulation of H^+-ATPase and membrane potential maintenance. This rules out voltage-gated outward-rectifying K^+ (GORK) channels as a downstream target in cross-talk between hypoxia and low-pH/Al stresses.

As the MIFE technique measures net ion fluxes, reduced net K^+ efflux may occur as a result of stronger unidirectional K^+ uptake. For the conditions of experiment (0.5 mM K^+ in the bath solution and MP values of less negative than -80 mV; **Figure 4.9**)

thermodynamically passive (channel-mediated) K^+ uptake will not be possible, and such unidirectional K^+ uptake should be mediated by some high-affinity K^+ uptake systems such as transporters from the KT/HAK/KUP family (Rodriguez-Navarro and Rubio, 2006). In barley, HvHAK1 transporter from this family is known to play a key role in high-affinity K^+ uptake (Rubio et al., 2000, Senn et al., 2001). The K^+ transporters of group I of the HAK/ KT/KUP family are suggested as major contributors to high-affinity K^+ uptake in several plant species, which consists of barley HAK1 (HvHAK1) and *Arabidopsis* HAK5 (AtHAK5) (Nieves-Cordones et al., 2010, Rodriguez-Navarro and Rubio, 2006). It is revealed that *AtHAK5* functions as the major component of the inducible high-affinity K^+ uptake system (Gierth et al., 2005, Pyo et al., 2010). *HvHAK1* gene, as a homologue of *AtHAK5*, is expressed exclusively in roots and induced when plants are starved for K (Rubio et al., 2000, Senn et al., 2001). HvHAK1 transporter plays a key role in high-affinity K^+ uptake in barley (Rubio et al., 2000, Senn et al., 2001) and studies with transgenic *Arabidopsis* plants expressing *HvHAK1* indicate a strong effect of K^+ deprivation on the contribution of HvHAK1 (Fulgenzi et al., 2008). However, we found that the mRNA levels of *HvHAK1* were significantly repressed by hypoxia **(Figure 4.9B)** and thus the transcriptional regulation of *HvHAK1* cannot explain the priming effects of hypoxia.

4.4.2 ROS production could contribute to priming effects of hypoxia

ROS in plants are formed as by-products of aerobic metabolism (Apel and Hirt, 2004). Under abiotic stress conditions, ROS levels are commonly elevated compared to pre-stress levels (Mittler, 2002). Excessive generation of ROS in root and leaf tissues was found during hypoxia and especially under re-oxygenation (Blokhina et al., 2003, Sairam et al., 2009, Shabala, 2011). Significant modification of the PM H^+-ATPase by H_2O_2 was reported for plants (Janicka-Russak and Kabala, 2012), and increased H_2O_2 production is considered to be one of the hallmarks of the low oxygen stress signal (Shabala et al., 2014). Al toxicity was also assumed to cause a rapid ROS production by mitochondria dysfunction (Yamamoto et al., 2002), formation of aluminium superoxide semi-reduced radicals (Exley, 2004), and activation of oxidising enzymes (Simonovicova et al., 2004). However, ROS over-accumulation is not considered as the primary mechanism for Al toxicity (Yamamoto et al., 2002). This could explain why

the priming effects of Al -treatment on ion homeostasis was not as efficient as hypoxia in perspective of ROS signalling **(Figure 4.8)**.

Depending on the circumstances, ROS may either exert toxic effects to plant cells or act as signalling molecules in a broad range of adaptive and developmental responses (Scholz-Starke et al., 2005). The relative abundance of the apoplastic H_2O_2, hydroxyl radicals and superoxide radicals has been considered to be a significant factor in integrating growth with stress responses (Demidchik et al., 2007). Similarly, in this study hypoxia-conditioned barley roots were shown to be less sensitive to ROS (H_2O_2) stress compared with non-conditioned plants, with significantly higher retention ability of K^+ in the EZ (**Figure 4.10A, 4.10E**). This can be attributed to 'non-deadly' effects of hypoxia stress for barley roots, as both the MP depolarization and K^+ loss could be fully recovered after 3-day hypoxia treatment (Zeng et al., 2014). This also strongly suggests that the ROS produced in roots during 3-d hypoxia has "primed" roots and desensitised ROS-activated K^+-permeable ion channels thus reducing root sensitivity to low-pH or Al stresses. On the other hand, it has been proven that altered ROS scavenging is highly efficient to alter ROS levels (Steffens et al., 2011). Thus, the priming effects of hypoxia pre-treatment may be attributed to the established ROS scavenging systems and enhanced capacity in tolerance to oxidative stresses. In regard to the two main components of ROS scavenging systems, it could be inferred that both of the activity of anti-oxidative enzyme and the synthesis of non-enzymatic antioxidant molecules increased upon hypoxia pre-treatment (Lee et al., 2007, Li et al., 2012, Logan et al., 2006). When taking the role of ROS in aluminium tolerance into consideration, this assumption could be supported by the previous report that the adverse effects caused by Al toxicity in *Brassica napus* was also found to be alleviated by over-expression of a manganese superoxide dismutase (Basu et al., 2001).

Apart from reduced oxygen availability, various phytotoxic secondary metabolites are accumulated in flooded soils (Armstrong and Gaynard, 1976, Pang et al., 2007, Shabala, 2011). Similar to conditioning with hypoxia, root priming in 4-hydroxybenzoic phenolic acid also reduced the root sensitivity to the combined low-pH/Al stress **(Figure 4.7)**. Indeed, the permeated phenolic acids could acidify both the apoplast once they disassociate (Pang et al., 2007, Shabala, 2011). Hence, we could conclude that this 'pre-adaptation' to low-pH environment may lead to the reduced sensitivity to the transient

low-pH/Al stresses. Although no direct evidence was provided for the ROS contribution in the priming effects of phenolic acid, the phenolic uptake when mediated by monocarboxylate transporters (MCT) was assumed to be able to increase the Ca^{2+}-mediated ROS production (Shabala, 2011). In our previous report, the net Ca^{2+} uptake was observed to be increased when roots were exposed to phenolics (Pang et al., 2007). The elevation of cytosol Ca^{2+} could activate the NADPH oxidase via positive feedback manners and consequently lead to a dramatic rise in ROS levels (Lecourieux et al., 2002).

4.4.3 The role of Ca^{2+} in stress cross-talk signalling

In the EZ of NC roots, the K^+ leak gradually increased with time, while the Ca^{2+} efflux was dominated temporarily and changed into influx and even remained higher than control values within 25 min after ROS treatment **(Figure 4.10A, 4.10E)**. A similar ion flux pattern was reported previously, when barley roots were exposed to waterlogging-associated phenolic acids treatment (Pang et al., 2007). This strongly suggests that the fluxes of these two ions were mediated by two different transport systems and could not be attributed to a general change in membrane permeability (Shabala, 2011). Recent studies have acknowledged the critical role of Ca^{2+} in oxidative stress signalling, as the induction of Ca^{2+} influx across the PM is central to ROS signalling (Pottosin et al., 2014, Shabala, 2011). It has been proposed that the addition of millimolar (mM) H_2O_2 could activate an EZ-PM hyperpolarization-activated Ca^{2+} channels (HACC) or depolarization-activated Ca^{2+} channels (DACC) (Demidchik et al., 2007, Shabala, 2011) leading to elevation of the cytosol Ca^{2+} levels and thus establishing related signalling for stress response (Demidchik et al., 2007, Rentel and Knight, 2004). In contrast, in the MZ of NC roots **(Figure 4.10F)**, the Ca^{2+} influx was short-lived and decreased rapidly to the initial level. Externally applied H_2O_2 was proposed not to be effective to induce Ca^{2+} influx until it converted to the hydroxyl radicals (OH·) in the MZ (Demidchik et al., 2007, Pottosin et al., 2014). Interestingly, when taking the hypoxia pre-treatment into consideration, the transiently profound influx of Ca^{2+} in EZ was not evident any more **(Figure 4.10E)**. This further supports our finding that the ROS accumulation during 3-day hypoxia treatment could act as an important switch for plant stress adaptation.

The Ca^{2+} efflux mechanisms also play a key role in forming the cytosolic Ca^{2+} signature (Bose et al., 2011). Dramatic efflux of Ca^{2+} induced by either the low-pH or combined low-pH /Al stress was observed in both NC and HC roots **(Figure 4.6)**. The Ca^{2+} -ATPase and Ca^{2+} exchangers (CAX, either with H^+ or Na^+) have been identified as two groups of Ca^{2+} efflux mechanisms (Bose et al., 2011). While no direct evidence for activity of Ca^{2+} -ATPase was included in our work, the massive H^+ influx **(Figure 4.5)** accompanied by Ca^{2+} efflux might explain how the roots deal with the shock of low-pH (4.0) or Al stress to maintain pH homeostasis (Zhao et al., 2008) and plasma MP. However, unlike the low-pH stress, the combined low-pH /Al stress caused nearly the same magnitude of Ca^{2+} efflux in either EZ or MZ and the priming effects of hypoxia was not evident. This was consistent with the previous observation that the Ca^{2+} flux changes did not differ among Al-resistant, Al-sensitive and the wild type *Arabidopsis* roots (Rincon-Zachary et al. 2010). Another possible reason is that the mechanisms underlie plant tolerance to low-pH and Al stresses appeared to be different (Bose et al., 2010a, Delhaize et al., 2012). In fact, there seems to be no stable pattern for Ca^{2+} kinetics under Al toxicity. While some studies found that Al could induce cytosolic Ca^{2+} rise in wheat and *Arabidopsis* roots (Rincon-Zachary et al., 2010, Zhang and Rengel, 1999), other studies showed that Al decreased the cytosolic Ca^{2+} concentration along with growth inhibition in tobacco cell cultures (Jones et al., 1998). Therefore, to get a better understanding of the Ca^{2+}efflux mechanisms, direct intracellular measurement of Ca^{2+} is required (Pottosin et al., 2014).

Chapter 5 Allelic variation of the major Al tolerance gene *HvAACT1* in barley [4]

5.1 Introduction

Aluminium (Al) is the most abundant metal in the earth crust. Al can be toxic to plants when the concentration of soluble Al increases in acidic soils (<pH 5.0) due to the formation of the phytotoxic Al^{3+} species. An early symptom of Al toxicity is the inhibition of root elongation which limits water and nutrient uptake (Kochian, 1995, Melakeberhan et al., 2001, Parker, 1995). The inhibition of root growth in bread wheat (*Triticum aestivum* L.) occurs within minutes or hours in simple hydroponic solutions due to decreases in root cell division and elongation (Amenos et al., 2009). Longer treatments result in thickened roots, damaged root cap, and lesions in the epidermal and cortical tissues near the root apices (Foy, 1984, Ryan et al., 1993, Zhou et al., 2013). Additional symptoms of stress in wheat and maize roots include the appearance of large swollen cortical cells near the root tip (Ciamporova, 2002, Zelinova et al., 2011).

An important mechanism of tolerance in many species relies on the exclusion of Al from root tissues by the release of organic anions from the root apices. The organic anions such as malate and citrate chelate the harmful Al cations in the apoplast and prevent Al damaging the root tissues (Delhaize et al., 2012, Maron et al., 2013). Barley (*Hordeum vulgare* L.) is one of the most Al-sensitive cereal species yet it still shows genotypic variation (Ma et al., 2004). A mechanism for Al tolerance described in barley relies on the Al-activated release of citrate from root apices. This is controlled by a single major locus *Alp* on chromosome 4HL (Furukawa et al., 2007, Ma et al., 2001, Wang et al., 2007). The gene underlying the *Alp* locus is *HvAACT1* which encodes a member of the multidrug and toxic compound extrusion (MATE) family (Ma et al., 2004, Ma et al., 2001, Ryan et al., 2001, Zhou et al., 2013). This gene has been linked with Al tolerance in many genotypes of barley including Murasakimochi, Dayton,

[4] This chapter has been submitted as: **Ma YL**, Li CD, Ryan P, Shabala S, You JF, Liu J, Liu CJ, Zhou MX. A novel allele for the aluminium tolerance gene in barley (*Hordeum vulgare* L.). BMC Genomics, 2016, In press (DOI: 10.1186/s12864-016-2551-3)

Honen, WB229, Svanhals, Br2 and Brindabella (Bian et al., 2013, Raman et al., 2002, Tang et al., 2000, Wang et al., 2006). Tolerant genotypes of barley show a constitutively higher expression of *HvAACT1* in root apices than sensitive ones. Furthermore, constitutively over-expression of *HvAACT1* in transgenic barley and wheat plants significantly increases the Al-activated citrate efflux and their tolerance to Al in hydroponic solution and in acid soil (Zhou et al., 2013).

The higher expression of *HvAACT1* in tolerant barley was recently linked to the presence of a 1023-bp transposable-element like insertion in the 5' untranslated region (UTR) of the gene *HvAACT1*. This insertion alters the usual distribution and level of *HvAACT1* expression such that it becomes constitutively high in the root apices (Delhaize et al., 2012, Fujii et al., 2012). This mutation is firstly found in cultivated Al-tolerant barley genotypes from East Asia where acid soils are prevalent and likely represents an important mutation that has helped the expansion of barley from the Near East where the soils are rarely acidic (Fujii et al., 2012).

A PCR-based marker can be used to detect the presence or absence of the insert in the 5'-UTR of *HvAACT1* and this is a convenient method for screening barley for Al tolerance (Bian et al., 2013, Fujii et al., 2012). The PCR product from tolerant barley genotypes is approximately 1 kb larger than the product from sensitive barley genotypes (Fujii et al., 2012). Another gene-specific marker called *HvMATE*-21 was designed to target polymorphism at the 3'-UTR of *HvAACT1* and this marker has been used successfully to score more than 50 genotypes differing in Al tolerance (Bian et al., 2013). All tolerant varieties tested possessed the 21-bp deletion compared with the sensitive ones. Using association analysis the *HvMATE*-21 marker could explain 66.9% of phenotypic variation for Al tolerance (Bian et al., 2013). Meanwhile, several simple sequence repeats (SSR) markers such as Bmac310, Bmag353 and HVM03 are closely linked with tolerance and routinely used for genetic analysis (Raman et al., 2003, Raman et al., 2002, Wang et al., 2007).

In this study, we identified a Chinese barley variety, CXHKSL, which was moderately tolerant to acid soil but which gave a non-standard result for the 5'-UTR marker of the *HvAACT1* gene. This indicated that Al tolerance in CXHKSL might be controlled or regulated in a different way. We investigated the Al tolerance mechanism in CXHKSL

and mapped the trait using a double haploid (DH) population derived from CXHKSL and the Al-sensitive variety Gairdner.

5.2 Materials and Methods

5.2.1 Plant materials

CXHKSL is a six-rowed Chinese variety that is tolerant of acid soils. The Al-sensitive variety, Gairdner, is an Australian malting barley. The Al tolerant variety, Dayton, was used as a control when investigating tolerance mechanisms of CXHKSL. One DH population consisting of 210 lines derived from a cross between CXHKSL and Gairdner was used for QTL mapping study.

Apart from CXHKSL and Gairdner, three tolerant varieties [including Honen and Brindabella (highly tolerant) andYSM1 (moderately tolerant)] and a sensitive variety (Baudin) were screened in the preliminary experiments. Similar with CXHKSL, YSM1 is a newly identified tolerant variety.

5.2.2 Experimental set-up for Al tolerance screening in soil conditions

Different growth conditions were explored to discriminate the six varieties effectively. Experiments were carried out in pots with three different soil types (pH= 4.3, 4.6, 5.0). Plants were grown under natural light during the day. Combined with three soil water contents **(Table 5.1)**, nine different growth conditions were compared. The trait of root length was used for Al tolerance assessment. To obtain similar root lengths at the seedling stage, the seeds were germinated on moist filter paper at 4 °C for 5 days. Seedlings with similar primary root lengths were transferred to pots. The pots were then placed in a glasshouse with 25°C/20°C day/night temperature with 16h photoperiod. They were grown under natural light during the day, supplemented by 400-W mercury lamps during morning and evening (PPFD 400 μmol/ (m^2·s)). Six to eight plants were taken for root length measurement for each variety after 7-day growing in acid soil with different pH values. Each replicate contains six varieties grown in a single pot (height 22cm × diameter 25cm). Every two pots were maintained in one black plastic tank

(55×35×22cm). Three different water levels (0cm, 6cm and 12 cm, respectively) were set up for each tank. Each growth condition was replicated twice. A control tank was set up with soil pH=5.8 in each replicate. Direct measurement of gravimetric water content of each soil moisture condition was taken **(Table 5.1)**. During each sample collection event, two 10-cm-long continuous soil cores of 5 cm in diameter were extracted from each pot after plants had been removed for root length measurement. All dry soil samples were obtained and oven-dried at 40°C for 72 hours. Soil nutrient composition of each soil type is shown in **Table 5.2**.

Table 5.1 Soil moisture corresponding to different water levels used in this study

Tank water levels (cm)	Gravimetric water content (w)[a] (%)
0 (Low)	18.75 ± 0.69
6 (Medium)	19.68 ± 1.22
12 (High)	21.47 ± 0.72

[a] Gravimetric water content (w)= $\frac{m_w}{m_d}$, m_w= mass/weight of water, m_d=mass/weight of dry soils

Table 5.2 Nutrient composition of three types of acid soils

Parameter	Type1	Type 2	Type 3
pH Level (in $CaCl_2$)	4.3	4.6	5
NH_4-N (mg/kg)	4	12	4
NO_3-N (mg/kg)	8	4	5
P (mg/kg)	14	11	9
K (mg/kg)	99	78	70
S (mg/kg)	13.1	12.4	5.6
Cu (mg/kg)	0.32	0.2	0.14
Fe (mg/kg)	63.91	64.7	32.42
Mg (mg/kg)	17.51	5.44	1.5
Zn (mg/kg)	30.48	0.87	0.47
Exchangeable Al (cmol/kg)	2.43	2.08	1.46
Other Exchangeable cations (cmol/kg)			
Ca	0.77	0.47	0.18
Na	0.27	0.09	0.05
Organic Carbon (%)	2.68	1.94	1.27
Conductivity (dS/m)	0.35	0.08	0.04

5.2.3 Al tolerance and root growth

The relative Al tolerance of the different varieties and selected double haploid lines (DHLs) were evaluated with hydroponic culture methods. Sterilized seeds were germinated in the dark for 2 days at 4 °C and then 2 days at 28°C. They were subsequently placed in an aerated nutrient solution containing 500 µM KNO_3, 500 µM $CaCl_2$, 500 µM NH_4NO_3, 150 µM $MgSO_4$, 10 µM KH_2PO4, 2 µM Fe:EDTA, 11 µM H_3BO_3, 2 µM $MnCl_2$, 0.35 µM $ZnCl_2$ and 0.2 µM $CuCl_2$. Al tolerance was estimated by measuring net root length after 4 days in 0, 1, and 4 µM $AlCl_3$ (pH=4.3), respectively. Relative root length (RRL) was estimated as: (net root growth in Al treatment/net root growth in control solution) × 100 % (Zhou et al., 2013). After 4-day growth, control and 4µM $AlCl_3$ -treated seedlings roots were stained with haematoxylin for 15 min and rinsed for 10 min to compare the density of Al accumulation at root apices. Haematoxylin could form a purple-red complex with Al and provides an indirect measurement of non-complex Al in root apices, with the intensity of staining correlated with sensitivity of Al toxicity (Delhaize et al., 2004).

Al tolerance was also scored in acid soil collected from the Northern Tasmania (pH=4.3). Three seeds of each DHL and parent varieties were sown in the acid soil in each replicate. Two independent experiments including six replicates were conducted in April and June 2013, respectively. Three replicates were applied in each experiment. Both root length and root morphology were used to assess Al tolerance. Root length (mm) of each seedling was measured seven days after sowing. Meanwhile, root tips were screened for the absence or presence of thickening caused by Al toxicity.

5.2.4 Assaying citrate efflux and malate efflux from root apices

Seedlings were grown for 4 days in the nutrient solution described above (without added $AlCl_3$). To study if the expression of *HvAACT1* needs longer Al treatment duration, a pre-treatment was carried out on half of the plants for each genotype by subjecting them to 0.2 mM $CaCl_2$ solution containing 10 µM $AlCl_3$ (pH=4.3) for overnight. Ten root apices (3-5 mm) with 4 replicates were excised from the same line and washed in 1 ml 0.2 mM $CaCl_2$ solution (pH= 4.3) on a platform shaker (60 rpm). After 30 min washing,

1 ml 0.2 mM $CaCl_2$ solution (pH= 4.3) with 30 μM $AlCl_3$ was added and shaken for 2 h at 60 rpm. The solutions were centrifuged to dryness on a rotary vacuum drier for citrate efflux detection. The enzyme assay used to determine citrate concentration is described by Wang *et al*. (2007). The initial citrate content in each sample was calculated from a standard curve. Malate concentration was measured with an enzyme assay as described previously (Ryan et al., 1995).

5.2.5 Molecular marker analysis

Three primer pairs were used to investigate allelic variation in the 5'UTR of *HvAACT1*. These were to detect the presence or absence of a ~1 kb transposon-like insertion previously described in Al-tolerant genotypes of barley. The first pair of primers was described by Fujii *et al.* (2012) with forward sequence 5'-GGTCCAACACTCTACCCTCCTT-3' and reverse 5'-GGTGCGAGTTGCCCCTA GCTATTA-3'. The second pair of primers was described by Bian *et al*. (2013) forward 5'-CTTCATTTCAACCAAGCACTCC-3' and reverse 5'-GCTTTTGGTCGAAC AAAGTATCG-3'. The third pair of primers was designed to amplify a slightly larger fragment that included the above two pairs of primers comprised (forward 5'-TGTCGATATGGTGCTCTTCG-3' and reverse 5'-AGCTCCATGACA ATTCTGGG-3'). PCR reactions were performed at 20μL-volume including 10 μL HotstarTaq^{TM} master mix (Qiagen), 2 μL primer mix (1:1 mix of forward and reverse primers at 10 nM), 3 μL DNA template, and 5 μL H_2O. Cycling conditions were as follows: 1 cycle of 1 min at 95^{o}C, 35 cycles of 1 min at 95^{o}C, 30 sec at 60^{o}C, 40 sec at 72^{o}C, and finally with an extension step of 1 min at 72^{o}C. All PCR reactions were run at C1000^{TM} Thermal cycler (BIO-RAD). PCR products were separated at 1% agarose and visualized by staining with 1% Red safe under Gel Doc^{TM} XR^{+} imagining system (BIO-RAD).

Together with three SSR markers (Bmac310, Bmag353 and HVM03) closely linked to the gene, (Bian et al., 2013, Ma et al., 2004, Wang et al., 2007), another *HvAACT1*-specific marker, *HvMATE*-21, was also used to genotype the population. *HvMATE*-21 was a PCR marker that detected the presence or absence of a 21-bp fragment in the 3'-UTR of *HvAACT1*. PCR reactions were carried out in a total volume of 15 μL containing

25~30 ng genomic DNA, 0.5 M of forward and reverse primers, 7.5 µL GoTaq® Hot Start Colorless Master Mix, 2X (Promega). The amplification of SSRs were performed by: 1 cycle of 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at the annealing temperature 55°C and 1 min at 72°C, with a final extension step of 5 min at 72°C. The PCR profiles for *HvMATE*-21 were almost the same as that for SSR markers except the annealing temperature was 60°C. All PCR reactions were run on Mastercycler Gradient 5331 (Eppendorf AG, Germany). The PCR products were separated on 5% denatured polyacrylamide gels and visualized by a rapid silver staining method (Liang et al., 2008).

5.2.6 Isolation and sequence analysis of coding region of HvACCT1 gene in CXHKSL and Dayton

The published complete coding DNA sequence (CDS) of *HvAACT1* gene (Genebank: AB302223.1) was retrieved from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nim.nih.gov/gene) and aligned with barley reference genome data using IPK blast server (http://webblast.ipk-gatersleben.de/barley/). Based on the best hit sequence, a total of 4 pairs of primers (**Table 5.3**) were used to amplify the whole *HvACCT1* open reading frames (ORFs). The amplified PCR products was purified and cloned with pGEM®-T Vector System (Promega). The final CDS were constructed using sequencing results from 12 independent clones (3 clones for each pair of primers). Sequence analysis was completed with software DNAMAN (version 7.0; Lynnon Biosoft, USA).

Table 5.3 List of primers used for isolations and sequencing coding regions of the *HvAACT1* gene

Primer name	Primer sequences
HvAACT1-g1F	5'-GATATGTGCCACCTTCGCTG-3'
HvAACT1-g1R	5'-CATCCCCAACCACTGCAAAA-3'
HvAACT1-g2F	5'-TCCGGGGCTTCAAAGATACA-3'
HvAACT1-g2R	5'-TGCTGCTGTGAAAGGGTCTA-3'
HvAACT1-g3F	5'-GCATCAACTTCGGAGCACAA-3'
HvAACT1-g3R	5'-AAGGGGACAGTGAGCTAACC-3'
HvAACT1-g4F	5'-CCTCTCTCTCAGGCAAGCAT-3'
HvAACT1-g4R	5'-TACCAGATGCGGGCAAATTG-3'

5.2.7 *HvAACT1* expression

RNA was isolated from root apices (also from plants used for citrate efflux measurement**)** by RNeasy^{TM} plant kit (Qiagen) and purified by inclusion of RNase-free DNase (Qiagen). One microgram total RNA was used to synthesize cDNA by reverse transcriptase system (Invitrogen). 1.0 μL oligo primer was added into 11.5 μL reaction mixture including 1 μg RNA. The mixture was incubated at 70^{o}C for 10 min, and transferred to ice immediately. Each aliquot included 4 μL buffer, 2 μL 0.1 M DTT, 1 μL dNTP mix, and 0.5 μL superscript III Reverse Transcriptase was added into the mixture, and incubated at 42^{o}C for 1 h. RNA degradation step was performed by addition of 0.25 μL RNase H (Thermo Scientific™) and incubated at 37^{o}C for 30 min.

Quantification real time polymerase chain reaction(RT-PCR) was run in a C1000TM Thermal cycler (BIO-RAD) with 10 μL reaction mixture containing 4.0 μL of cDNA diluted to 1:40, 5μL of SYBR Green Jumpstart Taq Readymix (Sigma) and 1 μL primer mix (1:1 mix of forward and reverse primers at 10 nM). Three pairs of primers were used to measure the expression of *HvAACT1* (including *HvAACT1*-forward 5'-AGCAGCCAAGACCTTGAGAA and reverse 5'-AGCAGGAATCCACAACCAAG-3'; New-*HvAACT1*-1-forward 5'-ACGGGGCTCTACCTCTTTGT-3' and reverse 5'-GGCAATAGAAACACCAACAGC-3'; New-*HvAACT1*-2-forward 5'-CTGTGTCA CTCTGGCATCGT-3', and reverse 5'-AAGCTGCAGAACACGAGAGGT-3'). The constitutively expressed barley glyceraldehydes -3-phosphate dehydrogenase

(HvGAPDH) gene and barley homologous to eukaryotic translation elongation factor 1A (HveEF-1A) gene were used as reference genes. The sequences of primers are as follows: *HvGAPDH*-forward: 5'-GTGAGGCTGGTGCTGATTACG-3' and reverse 5'-TGGTGCAGCTAGCATTTGAGAC-3', *HveEF-1A*-forward 5'-TTTCACTCTTG GTGTGAAGCAGAT-3' and reverse 5'-GACTTCCTTCACGATTTCTCGTAA-3'. Cycling conditions were 3 min at 95^{o}C, followed by 40 cycles at 95^{o}C for 10 sec, 60^{o}C for 20 sec, 68^{o}C for 10 sec. At the end, a melting curve of the amplified fragments was produced by increasing the temperature every 0.5°C from 60°C to 95°C.

5.2.8 Data analysis and QTL mapping

All phenotypic data was analysed using SPSS software package (Version 20.0, IBM), including all basic statistics calculation and Chi-Square Goodness of Fit Test. For genetic linkage map analysis, the genetic distances between molecular markers were calculated using software JoinMap 4.0 (Van Ooijen and Kyazma, 2006). The mean values of root lengths of DHLs were used to detect QTL affecting root tolerance to Al toxicity with software MapQTL6 (Van Ooijen and Kyazma, 2009). Interval mapping (IM) was first used to identify major QTL. By selecting significantly linked markers as cofactors, multiple QTL mapping (MQM) based on the multiple-QTL model was used. A set of 1000 permutations was performed to identify the LOD threshold corresponding to a genome-wide false discovery rate of 5% (P<0.05) (Van Ooijen and Kyazma, 2009).

5.3 Results

5.3.1 Preliminary experiments

5.3.1.1 Effects of different acid soil conditions on root growth

As the root lengths of all six varieties have no significant difference under control conditions (pH=5.8) (P<0.5) **(Table 5.4)**, root length data of varieties in acid soil was used for analysis. Variations in root length were observed under different soil moistures **(Table 5.1)** and pH treatments **(Table 5.2)**.

In soil with the lowest pH (4.3), the root length with high and medium soil moisture was shorter than that with the low soil moisture. Honen was the most tolerant variety (Group1) which was followed by Brindabella, CXHKSL and YSM1 (Group 2). In soil with the highest pH (5.0), the six varieties could be divided into three groups under high soil moisture, while no significant difference was observed among the six varieties under the treatment of the medium soil moisture. Unexpectedly CXHKSL was the most tolerant variety in the low soil moisture condition. The low soil moisture with soil pH=4.3 and high moisture with soil pH=5.0 gave ideal results, except Brindabella which was not shown as highly tolerant under those two conditions. In soil with pH=4.6 with low soil moisture, significant difference between highly tolerant (Honen and Brindabella) and medium tolerant varieties (CXHKSL and YSM1) was not detected. Significant difference in root length was also found between the sensitive variety (Baudin) and the tolerant varieties (CXHKL, YSM1 and Brindabella) in the treatments of high soil moisture. Meanwhile, compared with the medium soil moisture, both low and high soil moisture repressed the root elongation for two highly-tolerant varieties Honen and Brindabella.

Table 5.4 Root length of six varieties grown under non-acid soils (pH=5.8)

Varieties	Root length (Mean±SE)
Baudin	14.3±1.08
Brindabella	15.7±0.82
CXHKSL	14.0±0.71
Gairdner	14.0±0.65
Honen	14.3±2.04
YSM1	12.3±1.47

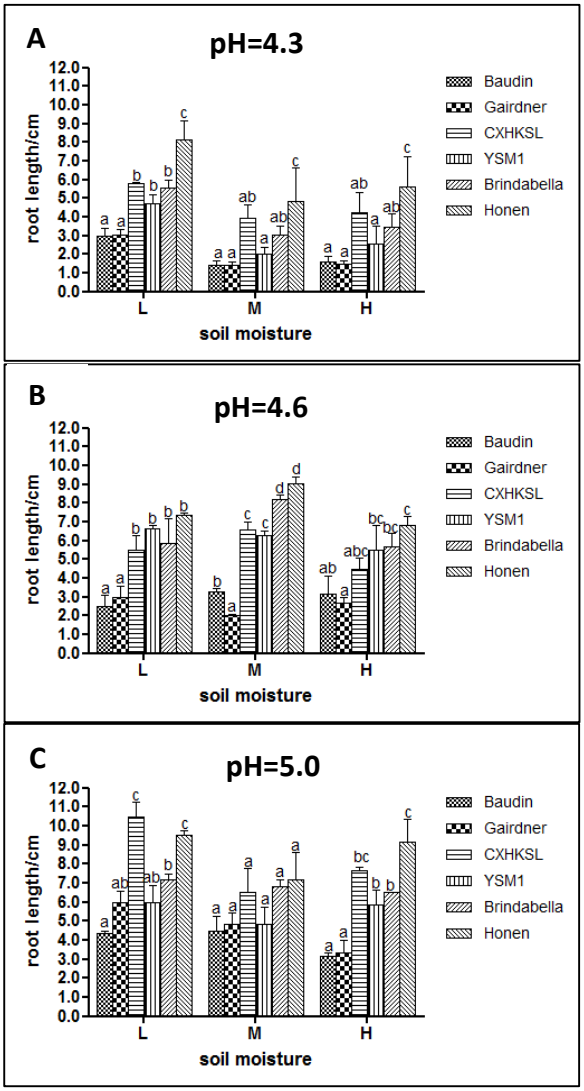

Figure 5.1 Root lengths under acid soils at (A) pH=4.3, (B) pH=4.6 and (C) pH=5.0 combined with three different soil moistures. 'L'= low water levels, 'M'=medium water levels and 'H'=high water levels. Different letters in indicate significant differences between groups based on one-way ANOVA (P≤0.05).

5.3.1.2 Effects of soil moisture on root growth in acid soils

To determine the suitable soil pH and soil moisture for Al tolerance screening experiments, the effects of soil moisture on Al tolerance were further assessed. The six varieties were initially divided into three groups according to the Al tolerance difference. In soil with pH=4.3, low soil moisture promoted root elongation compared with medium and high soil moisture in all three groups **(Figure 5.1A)**. In soil with pH=4.6, both low soil moisture and high soil moisture supressed the root growth significantly compared with medium soil moisture in the highly-tolerant group **(Figure 5.1B)**. In soil with pH=5.0, both the low and high soil moistures decreased the root length in highly-tolerant and medium tolerant groups. For the sensitive group, the root length was reduced as the soil moisture increased **(Figure 5.1C)**. Combined with the hydroponic culture method with the solution pH setting at 4.3, the set-up with pH=4.3 and low water moisture was finally selected for the Al tolerance screening in the soil experiments.

5.3.2 The identification of new allele by amplification of 5'-UTR of the *HvAACT1* gene

A PCR-based marker is available to score for the presence or absence of the ~1 kb insert in the 5'-UTR of the *HvAACT1* associated with higher expression in the root apices of Al-tolerant barley (Fujii et al., 2012). The marker generates a larger product in Al-tolerant genotypes than in sensitive genotypes. A Chinese variety named CXHKSL was identified to be tolerant to acid soil but did not show the typical marker result associated with tolerance. In fact the standard PCR reaction failed to produce any PCR band in CXHKSL while the expected fragments were obtained from control varieties including the highly tolerant variety Dayton and sensitive variety Gairdner **(Figure 5.2A)**. The failure to generate a fragment was not related to the quality or quantity of DNA extracted from CXHKSL since the same DNA sample was successful for various PCR amplification including the *HvMATE*-21 marker which targets the 3'-UTR of *HvAACT1* (data not shown). Polymorphisms in CXHKSL may have reduced annealing temperature of one or both primers and so two additional primer pairs were designed to target this polymorphic region in the 5'-UTR of *HvAACT1* **(Figure 5.2B, 5.2C)**. One set had primers farther upstream and downstream from the first set and therefore would amplify a slightly larger product **(Figure 5.2C)**. The second set of primers **(Figure 5.2B)**

was previously described by Bian *et al.* (2013). Using these additional primers, PCR products of the expected size were reliably generated from Dayton and Gairdner. However, neither of these primer pairs generated PCR products from CXHKSL **(Figure 5.2B, 5.2C)**. By contrast the *HvMATE*-21 marker for CXHKSL, Dayton and Gairdner were as described by Bian *et al.* (2013).

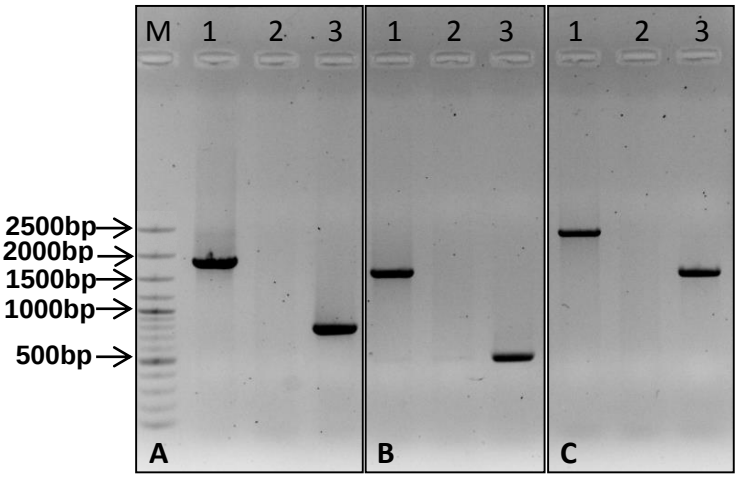

Figure 5.2 The 1-kb transposon-like insertion in the 5'-UTR of *HvAACT1* associated with Al tolerance. Three pairs of primers were used: (A) (B) (C) (see Materials and Methods). Varieties are Dayton (1), CXHKSL (2) and Gairdner (3).

5.3.3 The comparison of *HvAACT1* coding region sequences between CXHKSL and Dayton

The coding region of *HvAACT1* in both CXHKSL and Dayton consisted of 1,668 bp which is the same as previously published CDS (Furukawa et al., 2007). CXHKSL was similar to Dayton with only one nucleotide being different for *HvAACT1* coding region (**Figure 5.3**) and the difference didn't cause any changes in amino acids. Two SNPs were detected between the published CDS and CDS of CXHKSL and threee SNPs were detected between the CDS and CDS of Dayton.

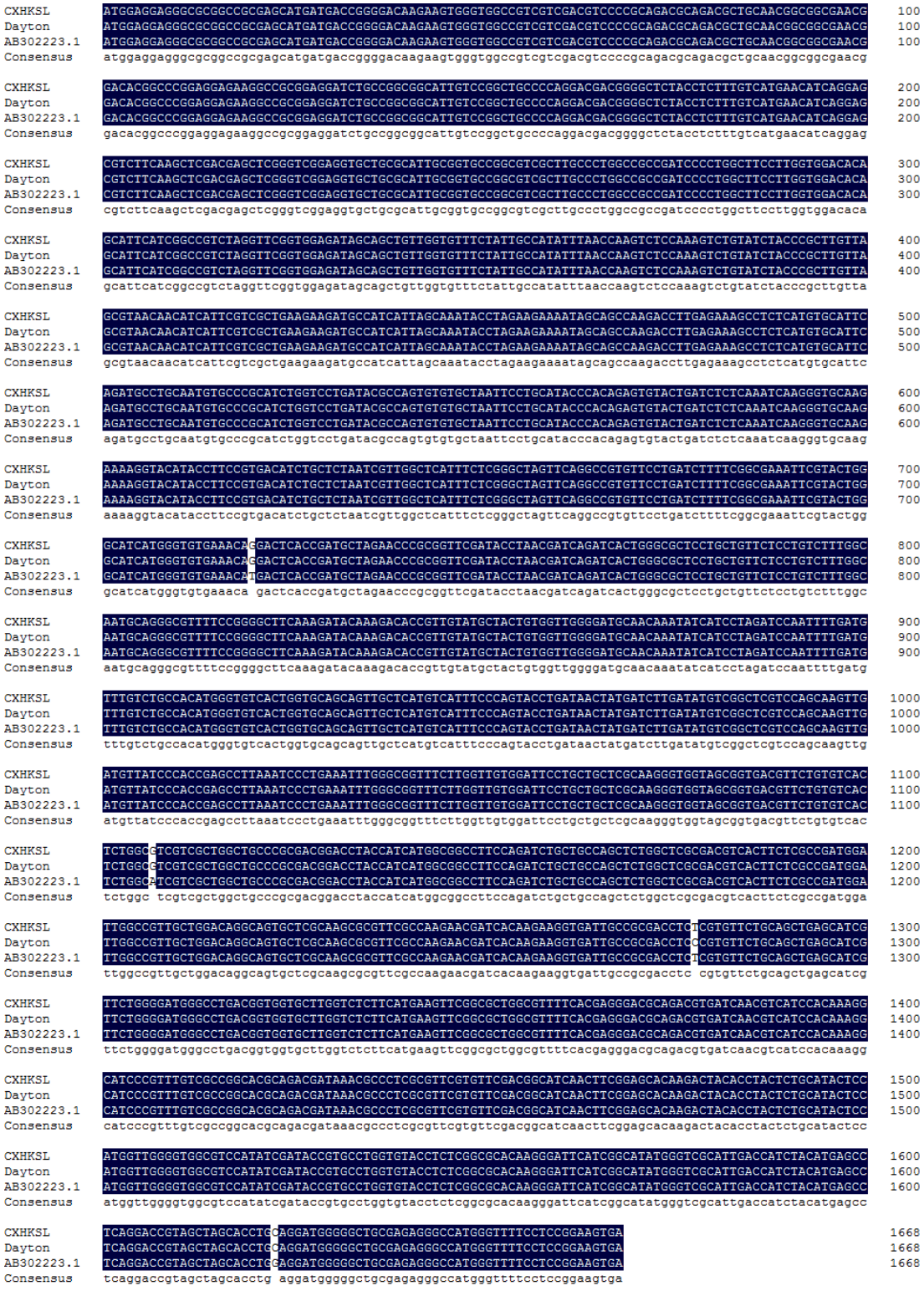

Figure 5.3 *HvAACT1* coding region in CXHKSL and Dayton. Bases with white background show SNPs.

5.3.4 Comparison of Al tolerance by measuring relative root length and citrate flux

The Al tolerance of CXHKSL was further investigated in hydroponic experiments and compared with those of Dayton and Gairdner. Relative root length (RRL) of CXHKSL was ~75% of the non-Al treated plants after four days in 4 µM $AlCl_3$ which was significantly greater than Gairdner (~30% RRL) but less than Dayton (~110% RRL) **(Figure 5.4)**. This ranking was similar to the Al-related tissue damage on roots among the three varieties **(Figure 5.5)**. Root apices of Gairdner became significantly thicker than those of the other two varieties after four days in 4 µM $AlCl_3$. Haematoxylin staining was also more intense in the root apices of Gairdner than in Dayton or CXHKSL, indicating greater Al accumulation in the roots of Gairdner. The Al-dependent efflux of citrate was then measured from these barley varieties. Citrate efflux from CXHKSL was less than that from Dayton (~40 $pmol \cdot apex^{-1} \cdot h^{-1}$) but greater than that from Gairdner which correlated well with their relative tolerance to Al **(Figure 5.6A)**. Pre-treatment with Al prior to these measurements did not increase citrate efflux further compared with roots without pre-treatment **(Figure 5.6A)**. These results indicate that the mechanism of Al tolerance in CXHKSL is associated with Al-activated citrate efflux from roots which is consistent with previous reports for barley (Ma et al., 2004, Wang et al., 2007). We also investigated whether Al-activated malate release from the roots of CXHKSL was apparent but found no indication of efflux from this in any tested barley genotypes (DHLs and varieties) **(Figure 5.6B)**. An Al-tolerant wheat line ET8 included as a positive control in this experiment showed malate efflux of 0.9 $nmol\ apex^{-1}h^{-1}$ which is similar to published values (Ryan et al., 1995).

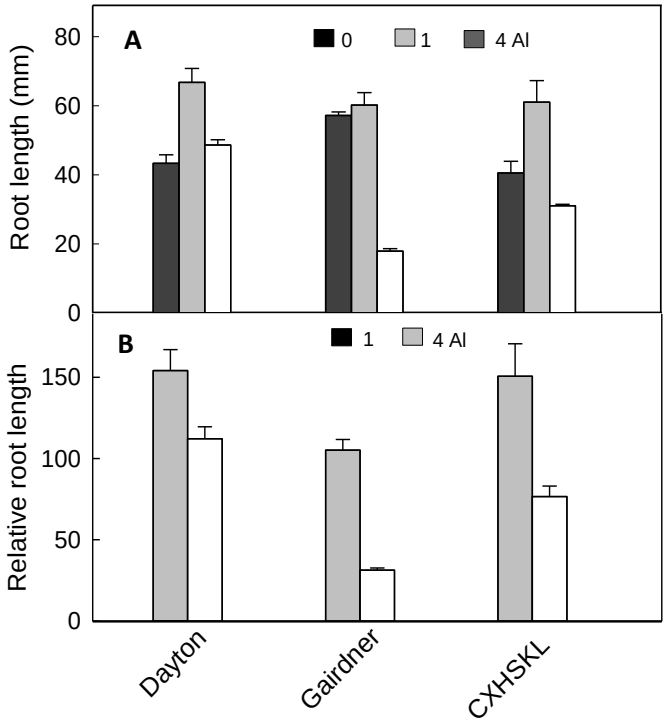

Figure 5.4 Comparison of Al tolerance among barley varieties in hydroponic culture. (A) Net root growth of seedlings after four days in the solution containing 0, 1 or 4 μM $AlCl_3$ (pH =4.3). (B) Relative root length at each Al concentration. Data showed means and standard error (SE) (n=4-7).

Figure 5.5 Comparison of haematoxylin staining of barley roots in hydroponic experiments following different treatments. Shown are root apices after 4 d in (A)

control solution and (B) 4 µM $AlCl_3$. Genotypes shown are Dayton (D), Gairdner (G) and CXHKSL (C). Darker staining indicated greater Al accumulation at the root apices.

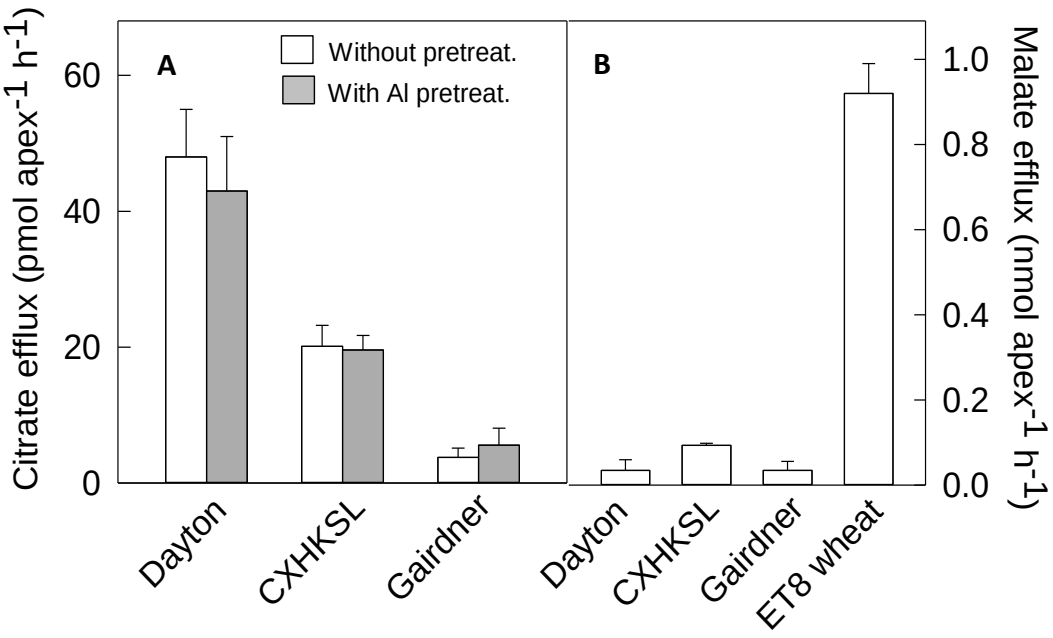

Figure 5.6 Citrate and malate efflux from the root apices of the barley varieties. (A) Citrate efflux was measured in presence of 30 µM $AlCl_3$ with and without an overnight pre-treatment in 10 µM $AlCl_3$. (B) Malate efflux measured in the presence of 30 µM $AlCl_3$ without pre-treatment. ET8 is an Al-tolerant wheat line used as a positive control for malate efflux. Data show means and SE (n=4).

5.3.4 QTL mapping of Al tolerance loci from CXHKSL

The chromosomal location of the tolerance locus in CXHKSL was investigated using a doubled haploid population generated by crossing CXHKSL and Gairdner. A total of 210 DHLs were grown in the acid soil and the roots were scored based on both root length and root tip damage (thickness). Root length of the CXHKSL parent was 75±8 mm and showed no damage of root tips **(Figure 5.7; Figure 5.8A).** Root length of the Gairdner was 40 ±5 mm and the root apices showed swelling, thickening and clear signs of damage **(Figure 5.7; Figure 5.8B)**. A total of 65 DHLs showed no thickened roots or visible tissue damage **(Figure 5.8C)** while 145 DHLs showed those strong phenotypes **(Figure 5.8D)**. When both criteria of growth and damage were used to score the population, we found that 128 DHLs had root lengths ≤ 50 mm and showed

thickened root apices and 52 DHLs had root lengths ≥70 mm without visible thickening of the root apices **(Figure 5.7; Figure 5.8)**. The remaining 30 DHLs had root lengths from 55 to 65 mm of which 17 DHLs showed thickening of the root apices **(Figure 5.8D)**.

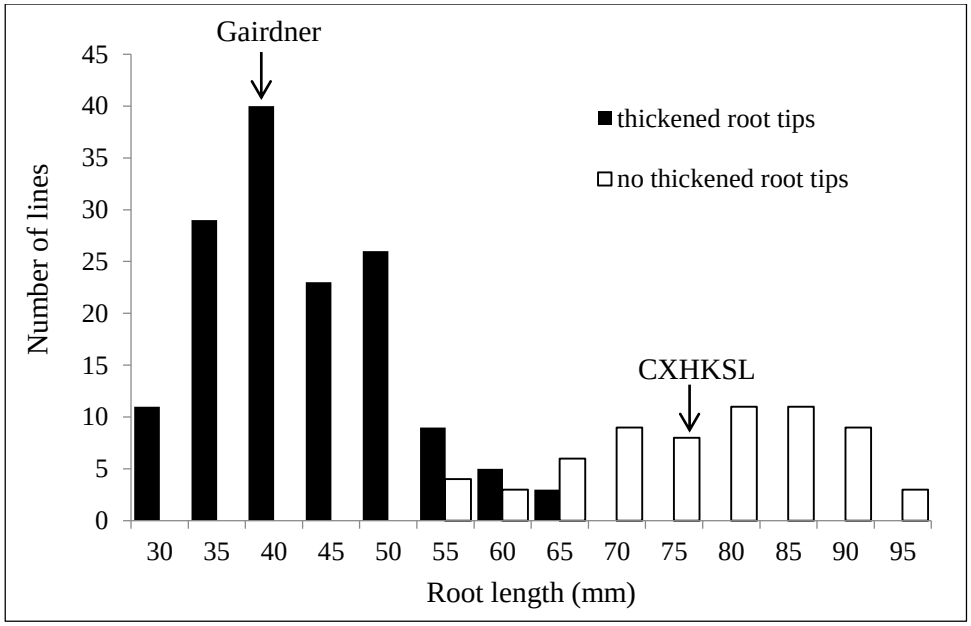

Figure 5.7 Distribution frequency of root length of 210 DHLs including parents after growth in an acid soil (pH=4.3). Solid bars indicated genotypes with thickened root tips and white bars indicate genotypes without thickened root tips.

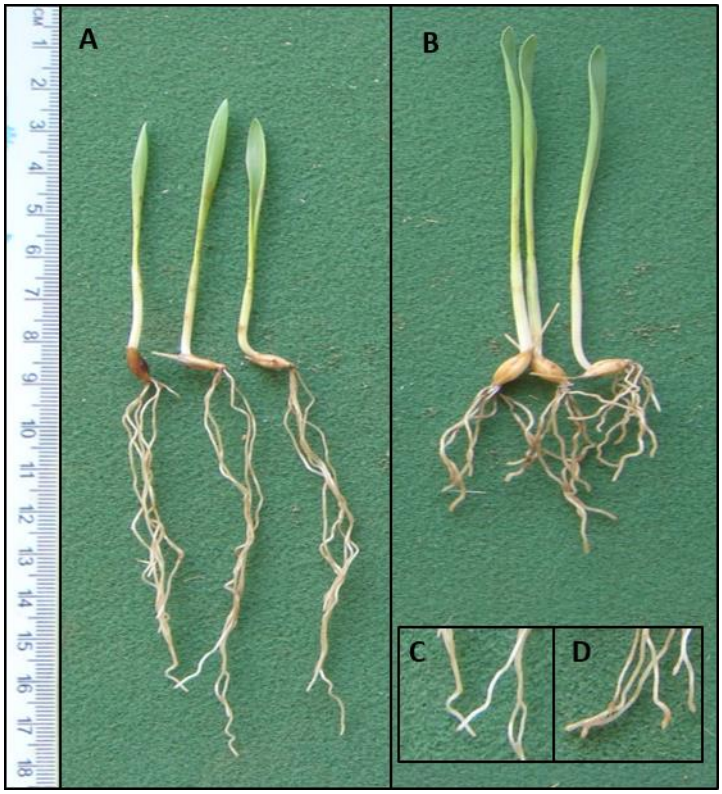

Figure 5.8 Comparison of Al tolerance of two parental varieties grown in acid soil (pH=4.3) by root length and damages of root tips. Root length was compared between (A) CXHKSL and (B) Gairdner. Typical root apices from an Al-tolerant genotype is shown in (C) and typical root apices from a sensitive genotype with obvious thickening and damage is show in (D).

Preliminary genetic analysis localised the Al tolerance phenotype in CXHKSL to a single major locus on chromosome 4H (data not shown). Therefore a more detailed genetic linkage map on chromosome 4H was generated using three SSR markers linked to Al tolerance (HVM03, Bmag353, Bmac310) as well as the *HvMATE*-21 marker which targets an insertion-deletion (INDEL) in the 3'UTR of *HvAACT1*. The map spanned a total length of 17.8cM and the order of markers **(Figure 5.9A)** was similar to that reported by Bian *et al*. (Bian et al., 2013). Analysis of root length under Al toxicity using this linkage map identified a significant QTL with a LOD score of 56.44 **(Figure 5.9B)**. The closest marker, *HvMATE*-21, accounted for 71.0% of the phenotypic variation, while Bmag353 and Bmac310 explained 61.0% and 50.5% of the variation respectively **(Figure 5.9B)**. We conclude that Al tolerance in CXHKSL maps to the *HvAACT1* gene as reported for other tolerant barley lines. This was further tested with

the *HvAACT1* 5'-UTR marker on a selection of tolerant and sensitive DHLs. All sensitive DHLs tested amplified a fragment similar to the parent Gairdner which is consistent with expectations. Similarly, all tolerant DHLs tested failed to produce the same fragment as CXHKSL (data not shown).

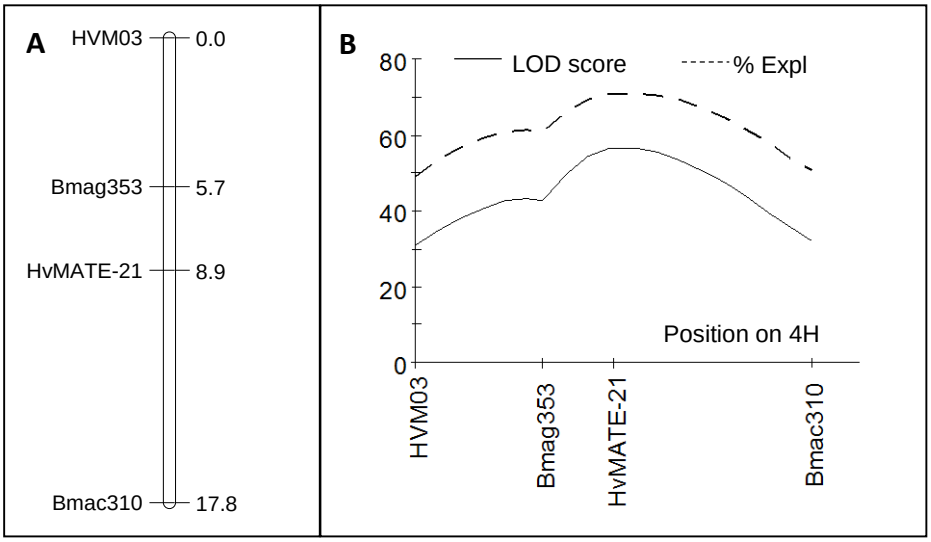

Figure 5.9 A-The linkage map on chromosome 4H with four molecular markers used to score 210 DHLs of the population CXHKSL/Gairdner. Numbers on the right side represented genetic distances in centiMorgan (cM); **B-QTL detected for Al tolerance on chromosome 4H using root length variation in acid soil**. The continuous line represented for the LOD score and the dashed line for phenotype variation (%) explained by each marker.

5.3.5 Comparison of citrate flux and *HvAACT1* expression among CXHKSL/ Gairdner DH lines

Citrate efflux was also measured from selected tolerant and sensitive DHLs. Six tolerant DHLs were examined and efflux was measured from all of them. Five of these had efflux of 18 to 28 pmol·apex^{-1}·h^{-1} which was similar to the parent CXHKSL **(Figure 5.10)**. Efflux from the remaining tolerant lines was greater than that from the sensitive lines measured which were <5 pmol·apex^{-1}·h^{-1} **(Figure 5.10)**. The level of *HvAACT1* expression was also determined in selected DHLs to determine whether this was linked

with tolerance measured by the other phenotypes of tolerance including citrate efflux. These measurements used two different reference genes *HvGAPDH* and *HveEF-1A*. Expression of *HvAACT1* was detected in CXHKSL, Dayton and the tolerant DHLs tested but no expression was detected in Gairdner or the three sensitive DHLs tested **(Figure 5.11)**. Moreover, three tolerant DH lines (DHL55, DHL224 and DHL222) even showed significantly higher expression levels than CXHKSL. These results suggest that Al-tolerance in CXHKSL is controlled by a novel allele of the *HvAACT1* gene.

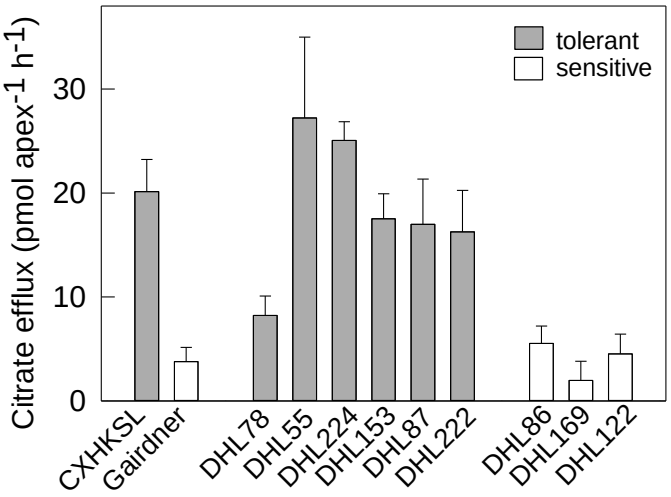

Figure 5.10 Citrate efflux from the root apices of the barley lines. Citrate efflux from the parental varieties CXHKSL and Gairdner as well as from Al tolerant and sensitive DHLs in the presence of 30 µM $AlCl_3$ without Al pre-treatment. Data showed means and SE (n=4).

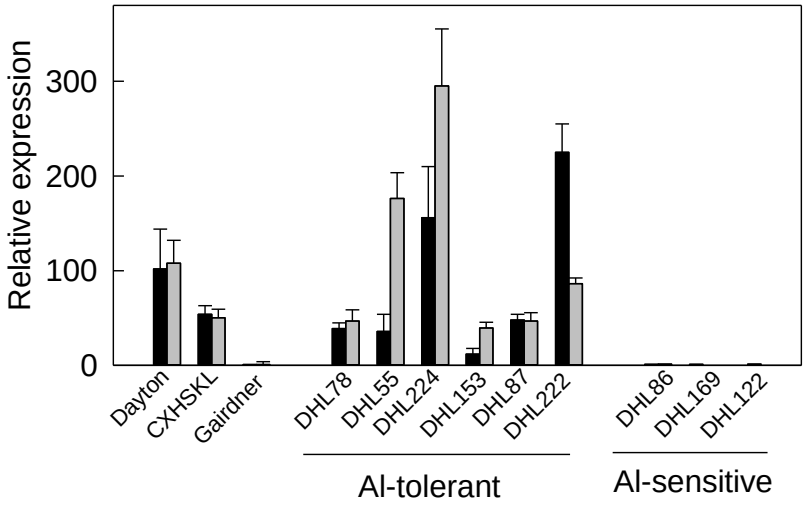

Figure 5.11 Relative expression of *HvAACT1* in Al-sensitive and Al-tolerant DHLs generated from Gairdner and CXHSKL measured with quantitative RT-PCR. The Al-tolerant variety Dayton was included. Expression in Gairdner was designated as 1.0. Data showed means of relative expression level using *HvGAPDH* (black bars) and *HveEF-1A* (shaded bars) as reference genes, and SE from three biological replicates with two technical replicates.

5.4 Discussion

The only mechanism of Al tolerance in barley described to date relies on the release of citrate from the root apices via the HvAACT1 transporter (Furukawa et al., 2007, Ma et al., 2004, Wang et al., 2007). In our study, we characterised a Chinese barley variety CXHKSL which is moderately tolerant to Al stress in hydroponics and acid soil but did not generate the expected result for a standard marker that targets the 1 kb insert in the 5'-UTR of *HvAACT1* correlated with Al tolerance. Dayton and Gairdner amplified fragments of expected sizes from the 5'-UTR of *HvAACT1* while no PCR products were detected in CXHKSL. The determinant role of promoter variation in Al tolerance was further confirmed by the fact that only one SNP was detected for CDS of *HvAACT1* gene between CXHKSL and Dayton without causing protein sequence changes. We showed that the Al tolerance mechanism in CXHKSL likely relies on the Al-activated efflux of citrate reported for other tolerant barley. Using a DH population generated by crossing CXHKSL and the Al-sensitive variety Gairdner and a set of markers linked with Al tolerance, we found that the tolerance locus in CXHKSL co-located to

HvAACT1. Collectively these results suggest that CXHKSL possesses a novel allele of Al-tolerance gene *HvAACT1*.

Fuji *et al.* (2012) reported that ~1 kb insertion in the upstream 5'-UTR of *HvAACT1* alters the distribution and level of gene expression in Al-tolerant varieties. We tried to detect the presence of this insert using the published pair of primers but no fragments were amplified in CXHKSL or the tolerant DH lines tested. However, the expression level of *HvAACT1* in CXHKSL, Dayton and Gairdner was positively correlated with the relative Al tolerance of these varieties with Dayton > CXHKSL > Gairdner. We also found that *HvAACT1* expression was higher in the tolerant DHLs than sensitive DHLs and that the marker targeting the 5'-UTR of *HvAACT1* segregated with tolerance (absence of a fragment in CXHKSL). This is consistent with the central role of *HvAACT1* in Al tolerance in these barley lines (Fujii et al., 2012). The absence of a PCR product with the 5'-UTR *HvAACT1* marker in CXHKSL could be due to sequence divergence which prevented primer binding to the DNA. We tested this possibility by designing the additional primers from the same region but these also failed to generate a product in CXHKSL **(Figure 5.2C)**. The absence of a PCR product for this marker could also be due to a deletion in CXHKSL or the presence of a much larger insertion. It is clear that polymorphisms exist in the 5'-UTR of *HvAACT1* in CXHKSL compared to the published sequences for other tolerant barley (Fujii et al., 2012).

The gene-specific marker *HvMATE*-21 was more efficient in predicting the phenotypic variation (71.0% in this study, **Figure 5.9**) under Al toxicity than the routinely-used SSR markers, Bmac310 and Bamag353 (Bian et al., 2013, Raman et al., 2002, Wang et al., 2007). Meanwhile, segregation distortion occurred in the DH population which skewed toward the sensitive parent Gairdner. No significant monogenic segregation ratio 1:1(χ^2 =36.82> $\chi^2_{0.05}$= 3.84) was observed. It is well-known that some loci in a genome often segregate in a non-random fashion (Lyttle, 1991). Such distortions can be caused by genes affecting gametic or hybrid fitness such as *Sd* genes in *Drosophila* (Temin and Marthas, 1984). Plasmotype may also influence the pollen selection to cause the segregation distortion (Liu et al., 1996).

Same as other tolerant barley genotypes, the main mechanism underlying Al tolerance in CXHKSL is secretion of citrate. The contribution of malate efflux to Al tolerance

was investigated in CXHKSL and other tolerant and sensitive genotypes but no significant malate release was detected. Al tolerance of wheat primarily relies on the Al-dependent malate efflux from root apices controlled by the Al-activated anion transporter encoded by the *TaALMT1* gene (Sasaki et al., 2004). The closest homologue of this gene in barley is *HvALMT1* which is located on chromosome 2H (Gruber et al., 2010). Although *HvALMT1* is not the major Al tolerance gene in barley, over-expression of *HvALMT1* gene in barley with a constitutive promoter can increase the efflux of malate and Al tolerance in barley and wheat (Gruber et al., 2011). Malate and citrate, as the most commonly released organic anions, can form harmless complexes with Al in the apoplast and protect the sensitive root apex and reduce uptake into the roots (Delhaize et al., 2012). This further supports that the Al tolerance mechanism in many species relies on the efflux of organic anions from root apices.

Chapter 6 General conclusions and future prospects

Waterlogging stress, salinity stress and aluminium (Al) toxicity are among the most potent abiotic stresses threatening cereal production in many parts of the world. Previous studies indicated that these abiotic stresses do not act alone. Rather, they can have strong interactions. For example, salinity stress can cause more severe damages in waterlogged soils (Setter and Waters, 2003, Xu et al., 2012) and soil acidification is often closely associated with waterlogging in high rainfall regions (Khabaz-Saberi and Rengel, 2010). It is also well understood that breeding and exploiting varieties with improved tolerance should be an integral part in minimizing damages from each of these abiotic stresses and a major gene conferring Al tolerance in barley, *HvAACT1*, has been identified (Ma et al., 2004, Wang et al., 2007). Aimed at further improving our understanding of tolerances to waterlogging, salinity and aluminium toxicity, the author identified QTL conferring salinity tolerance under waterlogged and drained conditions, investigated the tolerance mechanism of interacted hypoxia (the main adverse effect of short-term waterlogging) and low pH/Al stresses, and discovered novel allelic variation at the major Al tolerance gene *HvAACT1*.

6.1 Novel QTL conferring salinity tolerance under waterlogged and drained conditions

In this study, a double haploid (DH) population consisting of 175 lines, derived from a cross between a Chinese variety YSM1 and an Australian malting barley variety Gairdner, was used to construct a high density molecular map. Salinity tolerance of the parents and DH lines was evaluated under drained (SalinityD) and waterlogged (SalinityW) conditions in summer and winter times. It became clear during these assessments that adverse effects of salinity stress is dominant compared with the waterlogging stress. Three QTL located on chromosome 1H, a single QTL each located on chromosome 1H, 2H, 4H, 5H and 7H, respectively, were identified to be responsible for salinity tolerance under different environments. The two major QTL identified on chromosome 1H (*QSlsd.YG.1H*) for SalinityD (drained conditions) tolerance in the summer trial and the major QTL on chromosome 1H (*QSlww. YG.1H-1*) for SalinityW (waterlogged conditions) tolerance in the winter trial have never been reported before.

The QTL for salinity tolerance mapped on chromosomes 4H (*QSlwd.YG.4H*) and 7H (*QSlwd.YG.4H*, *QSlwd.YG.7H* and *QSlww.YG.7H*) were only identified in winter trials, while the QTL on chromosome 2H (*QSlsd.YG.2H* and *QSlsw.YG.2H*) were only detected in summer trials. In addition, significant effects of plant development stages (flowering time in this study) on plant salinity tolerance were detected. The significance levels of salinity tolerance QTL mapped on chromosome 2H and 5H was significantly reduced when the QTL analysis was conducted using flowering time as a covariate. By comparative mapping, it is found the QTL associated with flowering time were located at similar chromosome regions with two major genes controlling flowering time in barley, *VRN1* (chromosome 5H, a vernalisation-responsive gene) and *PPD-H1* (chromosome 2H, a photoperiod-responsive gene).

6.2 Tolerance mechanism of interacted hypoxia and low pH /Al stresses

We demonstrated, for the first time, the phenomenon of cross-protection between hypoxia and low-pH/Al stresses, and causally link it to cell's ability to maintain cytosolic K^+ homeostasis. Al toxicity is manifested only in acid conditions and it causes severe damages to the root system. Short-term waterlogging stress can occur simultaneously with Al toxicity in areas with high rainfall or inappropriate irrigation pattern. In this work, we used one waterlogging-sensitive cultivar Franklin and investigated effects of short-term treatments with hypoxia and phenolic acid (two major constraints in waterlogged soils) on roots' sensitivity to low-pH and Al stresses. We showed that hypoxia-primed roots maintained higher cell viability when exposed to low-pH/Al stress, in both elongation and mature root zones, and superior ability to retain K^+ in response to low-pH/Al stresses. These priming effects were not due to higher H^+-ATPase activity and better membrane potential maintenance, and could not be explained by the increased expression levels of *HvHAK1*, which mediates high-affinity K^+ uptake in roots. Instead, it is found that the reactive oxygen species (ROS) accumulation and signalling pathways mediated by Ca^{2+} could explain acclimation responses of plants to hypoxia and low-pH/Al stresses. Hypoxia-conditioned roots were significantly less sensitive to H_2O_2 treatment, indicated by the 10-fold reduction in the magnitude of K^+ efflux changes. This suggested that roots pre-treated with hypoxia desensitised ROS

(reactive oxygen species)-inducible K^+ efflux channels in root epidermis and had enhanced anti-oxidative capacity.

6.3 Novel allelic variation at the major Al tolerance gene locus *HvAACT1*

HvAACT1 is located on the same region as the *Alp* locus (the single dominant Al tolerance gene) on chromosome arm 4HL and has been designated as the major gene conferring Al tolerance in barley (Ma et al., 2004, Raman et al., 2002). The HvAACT1 protein facilitates the Al-activated release of citrate from root apices which protects the growing cells and enables root elongation to continue. A 1-kb transposable element-like insert in the 5'-UTR of *HvAACT1* is associated with increased gene expression and Al tolerance in Al-tolerant barley genotypes. PCR-based markers are developed to score for this insertion of 5'-UTR. We screened a wide range of barley genotypes for Al tolerance and identified a moderately tolerant Chinese genotype named CXHKSL which did not show the typical allele in the 5'-UTR of *HvAACT1* associated with tolerance. We investigated the mechanism of Al tolerance in CXHKSL and concluded it also relies on the Al-activated release of citrate from roots, which is the same as other Al-tolerant genotypes. QTL analysis of double haploid lines generated with CXHKSL and the Al-sensitive variety Gairdner mapped the tolerance locus to the same region as *HvAACT1* on chromosome 4H. We found that the Chinese barley genotype CXHKSL likely possesses a novel allele of the major Al tolerance gene *HvAACT1*.

6.4 Future prospects

The novel QTL for salinity tolerance under waterlogged and drained conditions, the tolerance mechanism for combined hypoxia and low pH/Al stresses and the allelic variation for the major Al tolerance gene identified in this project are valuable to stress-tolerance studies in barley. These findings improved the understandings in how plants respond to combined stresses and also increase the efficiency of barley breeding for high levels of stress tolerance. Research results from each of the three topics addressed in this PhD project provide novel opportunities including:

A. Develop diagnostic markers for improving the efficiency of breeding salinity tolerance varieties. Two novel QTL conferring salinity tolerance were identified in this study. However, due to the limited resolution of QTL mapping (Tuinstra et al., 1997), the available markers cannot be reliably used to tag genes underlying these QTL. One of the best ways of solving this problem is to create a series of near-isogenic lines (NILs) (Kaeppler et al., 1993, Ma et al., 2012). NIL-derived populations, segregating primarily for a targeted locus, allow the conversion of a quantitative trait into a Mendelian factor. Such populations make it possible to fine mapping a QTL. The release of the physical and functional sequence assembly of barley cultivar Morex (The International Barley Genome Sequencing Consortium, 2012) and a draft genome data of a Tibetan hulless barley genotype (*Hordeum vulgare* L. var. *nudum*) (Zeng et al., 2015) provides endless options in designing markers for any fine mapping effort. Moreover, the extensive synteny among cereal crops including rice, sorghum (*Sorghum bicolor*), *Brachypodium distachyon* and wheat should also facilitate the development of diagnostic markers by exploiting sequences from these different genomes (Drader and Kleinhofs, 2010, Hernandez et al., 2012, Mayer et al., 2011, Thiel et al., 2009). The development of diagnostic markers would not only significantly improve breeding efficiency via marker-assisted selection but also facilitate work on map-based gene cloning (Tanksley et al., 1995, Watanabe et al., 2009).

B. Investigate effects of other factors involved in the stress tolerance-associated ROS signalling. The vital roles of ROS signalling and ROS-associated Ca signalling in priming effects of hypoxia have been demonstrated in this study. Recently, several factors have been reported to participate in the regulation of ROS signalling and stress tolerance in model species. First, it has been proven that plant respiratory burst homolog (RBOHs) proteins perform a multitude of signalling functions in different assorted tissues (Suzuki et al., 2011), and coordinate the long-distance ROS signal transduction. Meanwhile, enhanced levels of Ca^{2+} in these abiotic stress-initiated cells can trigger enhanced ROS production by RBOH proteins to induce stress tolerance (Gilroy et al., 2014). Second, the hormones ABA and SA are involved in a broad range of stress tolerance, and their integration with ROS production has also been revealed (Drerup et al., 2013). Third, the enzymatic antioxidants are found to be able to

reduce the levels of excess ROS produced, alleviate the oxidative damages and maintain the beneficial role of ROS signalling in stress tolerance (Bose et al., 2014). To get a better understanding of the interaction between ROS and stress tolerance, the future work can focus on the three factors-associated metabolism: spatial coordination of ROS by RBOHs, hormone involvement in ROS signalling and functions of the anti-oxidative enzymes.

C. Breeding varieties with enhanced Al tolerance by gene pyramiding. A novel allele of the major Al tolerance gene *HvAACT1* was detected in this study. However, this locus conferred only partial resistance. As a wide genetic variation in Al tolerance exists in barley (Ma et al., 2004, Ma et al., 1997, Minella and Sorrells, 1992), additional loci conferring Al tolerance must exist. If several additional loci can be detected, it would be possible to breed novel varieties with high levels of Al tolerance by gene pyramiding as demonstrated in higher root growth on acid soil by specific combinations of *TaALMT1* and *TaMATE1B* alleles in wheat (Pereira et al., 2015), as well as resistances to the blast disease in rice (Fukuoka et al., 2015), crown rot in barley (Chen et al., 2015), the rust disease in wheat (Fu et al., 2009) and *Phytophthora sojae* in soybean (Nguyen et al., 2012).

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